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(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
24 February 2005 (24.02.2005)

PCT

(10) International Publication Number
WO 2005/016339 A1

- (51) International Patent Classification⁷: **A61K 31/425**, 31/50, 31/497, 31/44, 31/42
- (21) International Application Number:
PCT/US2003/023082
- (22) International Filing Date: 24 July 2003 (24.07.2003)
- (25) Filing Language: English
- (26) Publication Language: English
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- (84) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— with international search report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: METHODS FOR THE TREATMENT OF PARKINSON'S DISEASE

(57) Abstract: The present invention relates to methods and compositions for treating Parkinson's disease and for treating subjects susceptible to Parkinson's disease. In particular, the present invention provides agents that slow the progression of dopaminergic neuron loss in the substantia nigra, and that reduce the inflammatory responses of microglial cells and astrocytes in the substantia nigra.

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COMPOSITIONS AND METHODS FOR THE TREATMENT OF PARKINSON'S DISEASE

Field of the Invention

5 The present invention relates to methods and compositions for treating Parkinson's disease. In particular, the present invention provides agents that regulate the production of proinflammatory and neurotoxic products involved in Parkinson's disease.

10 Background of the Invention

 There are approximately 1.2 million persons in the United States and Canada living with PD and nearly 480 patients per million people are diagnosed each year with PD. The peak onset of PD is age 60 years, with the disease affecting roughly 1% of people over the age of 60 and nearly 2% of those aged 70 years and over.

15 However, PD is not just a disease of middle or old age: 15% of PD patients are 50 years of age or less, 10% are 40 years of age or less, and PD may also appear in teen-agers.

 Parkinson's disease is a disturbance of voluntary movement in which
20 muscles become stiff and sluggish, movement becomes clumsy and difficult and uncontrollable rhythmic twitching of groups of muscles produces characteristic shaking or tremor. The condition is believed to be caused by a degeneration of pre-synaptic dopaminergic neurons in the brain. Neuronal loss is accompanied by an inflammatory response mediated by microglia. This microglial-mediated focal
25 immune response then exacerbates the primary disease process and results in more extensive neuronal loss.

 It has been demonstrated that certain immunoregulatory cells, such as the microglia, possess the peroxisome proliferators-activated receptor- γ (PPAR γ).
30 PPAR γ is a member of the nuclear receptor superfamily that regulates carbohydrate and lipid metabolism. Recently, it has been shown that PPAR γ receptors participate in the inflammatory response and that, *in vivo*, PPAR γ agonists modulate

inflammatory responses in the brain (See M. Heneka et al., J. Neurosci. 20:6862-6867 [2000]).

5 Currently, the most widely used treatment for Parkinsonism is administration of L-DOPA, a precursor of dopamine which acts indirectly by replacing the missing dopamine. However, disadvantages are associated with the use of L-DOPA, for example, patients often suffer from side-effects such as dyskinesia and on-off effects, and it is necessary to administer L-DOPA in conjunction with a peripheral dopa-decarboxylase inhibitor such as carbidopa or benzaseride. Such treatment
10 improves quality of life for patients but does not halt disease progression. Furthermore, such treatment is associated with a number of adverse effects including nausea, vomiting, abdominal distension and psychiatric side-effects (for example, toxic confusional states, paranoia and hallucinations).

15 Thus far, the therapeutic strategies attempted have targeted neurotransmitter replacement, or the preservation of normal brain structures, which potentially provide short-time relief, but do not prevent neuronal degeneration and death. Therefore, there is a need for therapies that prevent neuronal degeneration and death associated with PD.

20

Summary of the Invention

 The present invention relates to a method of treatment of Parkinson's disease (PD) by the administration of a PPAR γ agonist, such as a thiazolidinedione, indole carboxylic acid derivative, or L-tyrosine-based compound. The present invention
25 provides a method of treatment for Parkinson's disease (PD) which comprises administering an effective non-toxic amount of such a therapeutic compound. In particular, the present invention provides agents that regulate the production of proinflammatory and neurotoxic products involved in PD.

30 The present invention provides methods for treating a subject, comprising administering a therapeutically effective amount of a PPAR γ agonist to the subject, e.g., wherein the subject is suffers from PD or is susceptible to PD.

The thiazolidinediones (TZDs) are a class of thiazolidine derivatives and known PPAR γ agonists. Additionally, new classes of PPAR γ agonists have been synthesized. One such class has been developed from naturally occurring L-tyrosine. A recent study demonstrated that a tyrosine based compound, GW7845,
5 was as effective as pioglitazone in preventing neurologic impairment in a murine model of experimental autoimmune encephalomyelitis (See D. Feinstein et al., Ann. Neurol. 51:694-702 [2002]). A second class of PPAR γ agonists recently developed includes a series of indole carboxylic acid derivatives. It has been shown that a series of 2,3-disubstituted indole 5-phenylacetic acid derivatives function as
10 selective PPAR γ agonists *in vivo* and that one compound from this series (i.e., 2-{3-[2-(4-methoxyphenyl)ethyl]-2-phenylindol-5-yl} acetic acid) has an *in vitro* potency at PPAR γ similar to or better than certain thiazolidinediones (See B. Henke et al., Biorg. Med. Chem. Lett. 9:3329-3334 [1999]).

15 In some embodiments of the present invention, the PPAR γ agonist comprises a thiazolidinedione, although all PPAR γ ligands and regulatory factors are contemplated by the present invention. In some embodiments, the thiazolidinedione comprises, but is not limited to, troglitazone, ciglitazone, pioglitazone, rosiglitazone (BRL 49653), englitazone, or combinations thereof. In other embodiments, the
20 PPAR γ agonist comprises, but is not limited to, docosahexaenoic acid, prostaglandin J₂ and prostaglandin J₂ analogs (e.g., ¹²-prostaglandin J₂ and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂).

In some embodiments of the present invention, the PPAR γ agonist comprises
25 an L-tyrosine-based compound, such as GW7845 or farglitazar, although all PPAR γ agonizing ligands and regulatory factors are contemplated by the present invention.

In yet another embodiment of the present invention, the PPAR γ agonist includes an indole-derived compound. In some embodiments, the indole-derived
30 compound comprises, but is not limited to, indole 5-carboxylic acid derivatives and 2,3-disubstituted indole 5-phenylacetic acid derivatives, although all PPAR γ ligands and regulatory factors are contemplated by the present invention.

In some embodiments of the present invention, the oral administration of thiazolidinediones and thiazolidinedione derivatives is contemplated, although, all administration means are also contemplated. In some embodiments, the

5 therapeutically effective amount of the PPAR γ agonist (i.e., thiazolidinedione or thiazolidinedione derivative) comprises approximately 10 mg/kg per day, although greater or lesser amounts are contemplated by the present invention.

Description of the Figures

10 FIGS. 1A-E show the effect of pioglitazone on MPTP-induced dopaminergic cell death: number of TH-positive neurons (a) in the SNpc of C57BL/6 mice treated with MPTP (4 · 15 mg/kg) in the absence (black) or presence (dark grey) of pioglitazone at day 2 (D2; n =10 per group), day 5 (D5; n= 5 per group) and day 8 (D8; n = 4–5 per group) post MPTP intoxication and in saline-injected (white) and

15 pioglitazonetreated (light grey) control animals, respectively. Data are presented as means \pm SEM (*p < 0.05 and **p < 0.01 compared with MPTP intoxicated animals; Newman–Keuls’ post-hoc analysis). (b–e) Photomicrographs of TH immunoreactivity in the SNpc of saline-injected control mice in the absence (b) or presence (c) of pioglitazone and of MPTP-treated mice at day 5 post MPTP in the

20 absence (d) or presence (e) of pioglitazone. There is a loss of TH-positive neurons and fibres in MPTP-treated mice (arrowheads). Scale bar represents 300 μ m (b–e).

FIGS. 2A-E show the absence of pioglitazone effect on MPTP-induced loss of TH-positive fibres in the striatum: striatal TH immunoreactivity determined by optical

25 densitometry (a) in MPTP-treated mice in the absence (black) or presence (dark grey) of pioglitazone at day 2 (D2; n $\frac{1}{4}$ 5 per group), day 5 (D5; n $\frac{1}{4}$ 5 per group), and day 8 (D8; n $\frac{1}{4}$ 4–5 per group) post MPTP injections, and in pioglitazone-treated mice (light grey) expressed as percentage of saline-injected controls. Data are presented as means \pm SEM (p-values between MPTP-treated animals and

30 controls were < 0.001 at all three time points; Newman–Keuls’ post-hoc analysis). (b–e) Photomicrographs of TH immunoreactivity in the striatum of salineinjected control mice in the absence (b) or presence (c) of pioglitazone, and of MPTPtreated

mice at day 5 post MPTP in the absence (d) or presence (e) of pioglitazone. Scale bar represents 400 μ m (b–e).

FIGS 3A–D show the levels of dopamine, DOPAC, HVA and noradrenaline in the striatum during MPTP intoxication. Striatal dopamine (a), dihydroxyphenylacetic acid (DOPAC; b), homovanillic acid (HVA; c), and noradrenaline (d) levels of MPTP intoxicated [two (D2), five (D5) and eight days (D8) post MPTP] and salineinjected mice (Co) under pioglitazone treatment (black bars) compared with mice that received no pioglitazone (grey bars). Data are presented as means \pm SEM (n = 5). The significance of the differences between control and MPTP-treated groups is indicated by symbols (*p < 0.05, **p < 0.01, and ***p < 0.001).

FIG. 3E shows the immunoblot detection of PPAR γ protein expression in ventral mesencephalon (vM) and striatum (ST) of pioglitazone and MPTP-treated mice. Murine adipose tissue served as positive control. The filter was stripped and reprobed with anti-actin antibody to confirm equal protein loading.

FIGS. 4A–J shows attenuation of MPTP-induced iNOS-positive, activated microglia in the SN in the presence of pioglitazone: Mac-1 immunoreactivity in MPTP-treated mice at day 2 (a), day 5 (c), and day 8 (e) post MPTP; in MPTP and pioglitazone-treated mice at day 2 (b), day 5 (d) and day 8 (f) post MPTP; and in saline-injected control mice in the absence (g) or presence (h) of pioglitazone. Magnification of Mac-1 immunostaining in MPTP-treated mice reveals typical morphology of activated microglial cells 2 days after MPTP injections (inset of panel a). iNOSpositive microglia in MPTP-treated animals (i) is absent in MPTP + pioglitazone-treated animals (j) at day 2 post MPTP. Scale bars represent 200 μ m (a–j). Insets of panel (a) and (i): 7 \times magnification of the respective panel.

FIG. 5A–H shows the MPTP-induced transient microglial activation remains unchanged in the striatum in the presence of pioglitazone: photomicrographs of Mac-1 immunostained microglia in MPTP-treated mice at day 2 (a), day 5 (c), and day 8 (e) post MPTP; in MPTP and pioglitazone-treated mice at day 2 (b), day 5 (d),

and day 8 (f) post MPTP; and in vehicle-treated control mice in the absence (g) or presence (h) of pioglitazone in the dorsal striatum. Scale bar represents 50 μ m (a–h).

FIG. 6A-H shows a reduction of MPTP-induced reactive astrocytosis in the mesencephalon in the presence of pioglitazone: glial fibrillary acidic protein (GFAP) immunostained astrocytes in the ventral mesencephalon of MPTP-treated mice at day 2 (a), day 5 (c), and day 8 (e) post MPTP; in MPTP and pioglitazone-treated mice at day 2 (b), day 5 (d), and day 8 (f) post MPTP; and in saline-injected control mice in the absence (g) or presence (h) of pioglitazone. Magnification of GFAP immunostaining in MPTP-treated mice shows typical morphology of reactive astrocytes at day 5 post MPTP (inset of panel c). Scale bar represents 200 μ m (a–h). Inset: 14 \times magnification of panel c.

FIG. 7A-H shows the MPTP-induced astrocytic response remains unchanged in the striatum in the presence of pioglitazone. Photomicrographs of glial fibrillary acidic protein (GFAP)-positive astrocytes in MPTP-treated mice at day 2 (a), day 5 (c), and day 8 (e) post MPTP; in MPTP and pioglitazone-treated mice at day 2 (b), day 5 (d) and day 8 (f) post MPTP; and in saline-injected control mice in the absence (g) or presence (h) of pioglitazone in the dorsal striatum. Scale bar represents 50 μ m (a–h).

FIGS. 8A-C shows the chemical structures of therapeutic compounds of the present invention.

FIG. 9 shows the chemical structures of therapeutic compounds of the present invention.

Definitions

To facilitate an understanding of the present invention, a number of terms and phrases are defined below:

As used herein, the term "therapeutically effective amount" refers to that amount of a composition that results in amelioration of symptoms or a

prolongation of survival in a patient. A therapeutically relevant effect relieves to some extent one or more symptoms of a disease or condition or returns to normal either partially or completely one or more physiological or biochemical parameters associated with or causative of the disease or condition.

5

As used herein, the term "PPAR γ agonist" refers to a compound or composition, which when combined with PPAR γ , directly or indirectly stimulates or increases an in vivo or in vitro reaction typical for the receptor (e.g., transcriptional regulation activity). The increased reaction can be measured by any of a variety of assays known to those skilled in the art. A preferred PPAR γ agonist is a
10 thiazolidinedione compound, including, but not limited to, troglitazone, BRL 49653, pioglitazone, ciglitazone, WAY-120,744, englitazone, AD 5075, darglitazone, and congeners, analogs, derivatives, and pharmaceutically acceptable salts thereof. A preferred PPAR γ agonist is an indole-based compound, including, but not limited to,
15 indole 5-carboxylic acid derivatives and 2,3-disubstituted indole 5-phenylacetic acid derivatives. Yet another preferred PPAR γ agonist is an L-tyrosine-based compound, including, but not limited to, GW7845 and farglitazar.

As used herein, the term "regulatory element" refers to a
20 deoxyribonucleotide sequence comprising the whole, or a portion of, an oligonucleotide sequence to which an activated transcriptional regulatory protein, or a complex comprising one or more activated transcriptional regulatory proteins, binds so as to transcriptionally modulate the expression of an associated gene or genes, including heterologous genes.

25

As used herein, the term "transcriptional regulatory protein" refers to cytoplasmic or nuclear proteins that, when activated, bind the regulatory elements/oligonucleotide sequences of the present invention either directly, or indirectly through a complex of transcriptional regulatory proteins or other adapter
30 proteins, to transcriptionally modulate the activity of an associated gene or genes. Thus, transcriptional regulatory proteins can bind directly to the DNA regulatory elements of the present invention, or can bind indirectly to the regulatory elements

by binding to another protein, which in turn binds to or is bound to a DNA regulatory element of the present invention.

As used herein, the term "transcriptionally modulate the expression of an associated gene or genes" means to change the rate of transcription of such gene or genes.

As used herein, the term "central nervous system" refers to all structures within the dura mater. Such structures include, but are not limited to, the brain and spinal cord.

As used herein, the terms "host" and "subject" refer to any animal, including, but not limited to, humans and non-human animals (e.g., rodents, arthropods, insects [e.g., Diptera], fish [e.g., zebrafish], non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.), which is to be the recipient of a particular treatment. Typically, the terms "host," "patient," and "subject" are used interchangeably herein in reference to a human subject. As used herein, the term "subject suffering from Parkinson's disease" refers to subjects that are identified as having or likely having Parkinson's disease. As used herein the terms "subject susceptible to Parkinson's disease" refer to subjects identified as having a risk of contracting or developing Parkinson's disease.

As used herein, the term "non-human animals" refers to all non-human animals. Such non-human animals include, but are not limited to, vertebrates such as rodents, non-human primates, ovines, bovines, ruminants, lagomorphs, porcines, caprines, equines, canines, felines, aves, etc.

The term "biologically active," as used herein, refers to a protein or other biologically active molecules (e.g., catalytic RNA) having structural, regulatory, or biochemical functions of a naturally occurring molecule.

The term "agonist," as used herein, refers to a molecule which, when interacting with a biologically active molecule, causes a change (e.g., enhancement) in the biologically active molecule, which modulates the activity of the biologically active molecule. Agonists include, but are not limited to proteins, nucleic acids, carbohydrates, lipids or any other molecules which bind or interact with biologically active molecules. For example, agonists can alter the activity of gene transcription by interacting with RNA polymerase directly or through a transcription factor or signal transduction pathway.

The terms "antagonist" or "inhibitor," as used herein, refer to a molecule which, when interacting with a biologically active molecule, blocks or modulates the biological activity of the biologically active molecule. Antagonists and inhibitors include, but are not limited to, proteins, nucleic acids, carbohydrates, lipids or any other molecules that bind or interact with biologically active molecules. Inhibitors and antagonists can affect the biology of entire cells, organs, or organisms (e.g., an inhibitor that slows or prevents neuronal degeneration and death).

The term "modulate," as used herein, refers to a change in the biological activity of a biologically active molecule. Modulation can be an increase or a decrease in activity, a change in binding characteristics, or any other change in the biological, functional, or immunological properties of biologically active molecules.

As used herein, the term "nucleic acid molecule" refers to any nucleic acid containing molecule including, but not limited to DNA or RNA. The term encompasses sequences that include any of the known base analogs of DNA and RNA including, but not limited to, 4-acetylcytosine, 8-hydroxy-N6-methyladenosine, aziridinylcytosine, pseudoisocytosine, 5-(carboxyhydroxymethyl)uracil, 5-fluorouracil, 5-bromouracil, 5-carboxymethylaminomethyl-2-thiouracil, 5-carboxymethylaminomethyluracil, dihydrouracil, inosine, N6-isopentenyladenine, 1-methyladenine, 1-methylpseudouracil, 1-methylguanine, 1-methylinosine, 2,2-dimethylguanine, 2-methyladenine, 2-methylguanine, 3-methylcytosine, 5-methylcytosine, N6-methyladenine, 7-methylguanine, 5-methylaminomethyluracil,

5-methoxyaminomethyl-2-thiouracil, beta-D-mannosylqueosine, 5'-methoxycarbonylmethyluracil, 5-methoxyuracil, 2-methylthio-N6-isopentenyladenine, uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, oxybutoxosine, pseudouracil, queosine, 2-thiocytosine, 5-methyl-2-thiouracil, 2-thiouracil, 4-thiouracil, 5-methyluracil, N-uracil-5-oxyacetic acid methylester, uracil-5-oxyacetic acid, pseudouracil, queosine, 2-thiocytosine, and 2,6-diaminopurine.

The term "gene" refers to a nucleic acid (e.g., DNA) sequence that comprises coding sequences necessary for the production of a polypeptide or precursor. The polypeptide can be encoded by a full length coding sequence or by any portion of the coding sequence so long as the desired activity or functional properties (e.g., enzymatic activity, ligand binding, signal transduction, etc.) of the full-length or fragment is retained. The term also encompasses the coding region of a structural gene and the including sequences located adjacent to the coding region on both the 5' and 3' ends for a distance of about 1 kb or more on either end such that the gene corresponds to the length of the full-length mRNA. The sequences that are located 5' of the coding region and which are present on the mRNA are referred to as 5' non-translated sequences. The sequences that are located 3' or downstream of the coding region and which are present on the mRNA are referred to as 3' non-translated sequences. The term "gene" encompasses both cDNA and genomic forms of a gene. A genomic form or clone of a gene contains the coding region interrupted with non-coding sequences termed "introns" or "intervening regions" or "intervening sequences." Introns are segments of a gene which are transcribed into nuclear RNA (hnRNA); introns may contain regulatory elements such as enhancers. Introns are removed or "spliced out" from the nuclear or primary transcript; introns therefore are absent in the messenger RNA (mRNA) transcript. The mRNA functions during translation to specify the sequence or order of amino acids in a nascent polypeptide.

As used herein, the term "gene expression" refers to the process of converting genetic information encoded in a gene into RNA (e.g., mRNA, rRNA, tRNA, or snRNA) through "transcription" of the gene (i.e., via the enzymatic action

of an RNA polymerase), and for protein encoding genes, into protein through "translation" of mRNA. Gene expression can be regulated at many stages in the process. "Up-regulation" or "activation" refers to regulation that increases the production of gene expression products (i.e., RNA or protein), while "down-regulation" or "repression" refers to regulation that decrease production. Molecules (e.g., transcription factors) that are involved in up-regulation or down-regulation are often called "activators" and "repressors," respectively.

Where "amino acid sequence" is recited herein to refer to an amino acid sequence of a naturally occurring protein molecule, "amino acid sequence" and like terms, such as "polypeptide" or "protein" are not meant to limit the amino acid sequence to the complete, native amino acid sequence associated with the recited protein molecule.

In addition to containing introns, genomic forms of a gene may also include sequences located on both the 5' and 3' end of the sequences which are present on the RNA transcript. These sequences are referred to as "flanking" sequences or regions (these flanking sequences are located 5' or 3' to the non-translated sequences present on the mRNA transcript). The 5' flanking region may contain regulatory sequences such as promoters and enhancers that control or influence the transcription of the gene. The 3' flanking region may contain sequences which direct the termination of transcription, post-transcriptional cleavage and polyadenylation.

The term "wild-type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designed the "normal" or "wild-type" form of the gene. In contrast, the term "modified" or "mutant" refers to a gene or gene product which displays modifications in sequence and or functional properties (i.e., altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated; these are identified by the fact that

they have altered characteristics when compared to the wild-type gene or gene product.

As used herein, the term "oligonucleotide," refers to a short length of single-stranded polynucleotide chain. Oligonucleotides are typically less than 100 residues long (e.g., between 15 and 50), however, as used herein, the term is also intended to encompass longer polynucleotide chains. Oligonucleotides are often referred to by their length. For example a 24 residue oligonucleotide is referred to as a "24-mer". Oligonucleotides can form secondary and tertiary structures by self-hybridizing or by hybridizing to other polynucleotides. Such structures can include, but are not limited to, duplexes, hairpins, cruciforms, bends, and triplexes.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. The term "vehicle" is sometimes used interchangeably with "vector." Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

The term "expression vector" as used herein refers to a recombinant DNA molecule containing a desired coding sequence and appropriate nucleic acid sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Nucleic acid sequences necessary for expression in prokaryotes usually include a promoter, an operator (optional), and a ribosome binding site, often along with other sequences. Eukaryotic cells are known to utilize promoters, enhancers, and termination and polyadenylation signals.

Transcriptional control signals in eukaryotes comprise "promoter" and "enhancer" elements. Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription (T. Maniatis et al., Science 236:1237 [1987]). Promoter and enhancer elements have been isolated from a variety of eukaryotic sources including genes in yeast, insect and mammalian cells, and viruses (analogous control elements, i.e., promoters, are also found in prokaryote). The selection of a particular promoter and enhancer

depends on what cell type is to be used to express the protein of interest. Some eukaryotic promoters and enhancers have a broad host range while others are functional in a limited subset of cell types (for review see, S. D. Voss et al., Trends Biochem. Sci., 11:287 [1986]; and T. Maniatis et al., supra). For example, the SV40 early gene enhancer is very active in a wide variety of cell types from many mammalian species and has been widely used for the expression of proteins in mammalian cells (R. Dijkema et al., EMBO J. 4:761 [1985]). Two other examples of promoter/enhancer elements active in a broad range of mammalian cell types are those from the human elongation factor 1.alpha. gene (T. Uetsuki et al, J. Biol. Chem., 264:5791 [1989]; D. W. Kim et al., Gene 91:217 [1990]; and S. Mizushima and S. Nagata, Nuc. Acids. Res., 18:5322 [1990]) and the long terminal repeats of the Rous sarcoma virus (C. M. Gorman et al., Proc. Natl. Acad. Sci. USA 79:6777 [1982]) and the human cytomegalovirus (M. Boshart et al, Cell 41:521 [1985]). Some promoter elements serve to direct gene expression in a tissue-specific manner.

As used herein, the term "promoter/enhancer" denotes a segment of DNA which contains sequences capable of providing both promoter and enhancer functions (i.e., the functions provided by a promoter element and an enhancer element, see above for a discussion of these functions). For example, the long terminal repeats of retroviruses contain both promoter and enhancer functions. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one which is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one which is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques such as cloning and recombination) such that transcription of that gene is directed by the linked enhancer/promoter.

The presence of "splicing signals" on an expression vector often results in higher levels of expression of the recombinant transcript. Splicing signals mediate the removal of introns from the primary RNA transcript and consist of a splice donor and acceptor site (J. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, New York [1989], pp. 16.7-16.8). A

commonly used splice donor and acceptor site is the splice junction from the 16S RNA of SV40.

Efficient expression of recombinant DNA sequences in eukaryotic cells requires expression of signals directing the efficient termination and polyadenylation of the resulting transcript. Transcription termination signals are generally found downstream of the polyadenylation signal and are a few hundred nucleotides in length. The term "poly A site" or "poly A sequence" as used herein denotes a DNA sequence that directs both the termination and polyadenylation of the nascent RNA transcript. Efficient polyadenylation of the recombinant transcript is desirable as transcripts lacking a poly A tail are unstable and are rapidly degraded. The poly A signal utilized in an expression vector may be "heterologous" or "endogenous." An endogenous poly A signal is one that is found naturally at the 3' end of the coding region of a given gene in the genome. A heterologous poly A signal is one that is isolated from one gene and placed 3' of another gene. A commonly used heterologous poly A signal is the SV40 poly A signal. The SV40 poly A signal is contained on a 237 bp BamHI/BclI restriction fragment and directs both termination and polyadenylation (J. Sambrook, *supra*, at 16.6-16.7).

Eukaryotic expression vectors may also contain "viral replicons" or "viral origins of replication." Viral replicons are viral DNA sequences that allow for the extrachromosomal replication of a vector in a host cell expressing the appropriate replication factors. Vectors that contain either the SV40 or polyoma virus origin of replication replicate to high "copy number" (up to 104 copies/cell) in cells that express the appropriate viral T antigen. Vectors that contain the replicons from bovine papillomavirus or Epstein-Barr virus replicate extrachromosomally at "low copy number" (~100 copies/cell).

The term "homology" refers to a degree of complementarity. There may be partial homology or complete homology (i.e., identity). A partially complementary sequence is one that at least partially inhibits a completely complementary sequence from hybridizing to a target nucleic acid is referred to using the functional term

"substantially homologous." The inhibition of hybridization of the completely complementary sequence to the target sequence may be examined using a hybridization assay (Southern or Northern blot, solution hybridization and the like) under conditions of low stringency. A substantially homologous sequence or probe will compete for and inhibit the binding (i.e., the hybridization) of a completely homologous to a target under conditions of low stringency. This is not to say that conditions of low stringency are such that non-specific binding is permitted; low stringency conditions require that the binding of two sequences to one another be a specific (i.e., selective) interaction. The absence of non-specific binding may be tested by the use of a second target that lacks even a partial degree of complementarity (e.g., less than about 30% identity); in the absence of non-specific binding the probe will not hybridize to the second non-complementary target.

The art knows well that numerous conditions may be employed to comprise low stringency conditions; factors such as the length and nature (DNA, RNA, base composition) of the probe and nature of the target (DNA, RNA, base composition, present in solution or immobilized, etc.) and the concentration of the salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol) are considered and the hybridization solution may be varied to generate conditions of low stringency hybridization different from, but equivalent to, the above listed conditions. In addition, the art knows conditions that promote hybridization under conditions of high stringency (e.g., increasing the temperature of the hybridization and/or wash steps, the use of formamide in the hybridization solution, etc.) (see definition below for "stringency").

When used in reference to a double-stranded nucleic acid sequence such as a cDNA or genomic clone, the term "substantially homologous" refers to any probe that can hybridize to either or both strands of the double-stranded nucleic acid sequence under conditions of low stringency as described above.

A gene may produce multiple RNA species that are generated by differential splicing of the primary RNA transcript. cDNAs that are splice variants of the same

gene will contain regions of sequence identity or complete homology (representing the presence of the same exon or portion of the same exon on both cDNAs) and regions of complete non-identity (for example, representing the presence of exon "A" on cDNA 1 wherein cDNA 2 contains exon "B" instead). Because the two

5 cDNAs contain regions of sequence identity they will both hybridize to a probe derived from the entire gene or portions of the gene containing sequences found on both cDNAs; the two splice variants are therefore substantially homologous to such a probe and to each other.

10 When used in reference to a single-stranded nucleic acid sequence, the term "substantially homologous" refers to any probe that can hybridize (i.e., it is the complement of) the single-stranded nucleic acid sequence under conditions of low stringency as described above.

15 As used herein, the term "hybridization" is used in reference to the pairing of complementary nucleic acids. Hybridization and the strength of hybridization (i.e., the strength of the association between the nucleic acids) is impacted by such factors as the degree of complementary between the nucleic acids, stringency of the conditions involved, the T_m of the formed hybrid, and the G:C ratio within the

20 nucleic acids. A single molecule that contains pairing of complementary nucleic acids within its structure is said to be "self-hybridized."

As used herein, the term " T_m " is used in reference to the "melting temperature." The melting temperature is the temperature at which a population of

25 double-stranded nucleic acid molecules becomes half dissociated into single strands. The equation for calculating the T_m of nucleic acids is well known in the art. As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G+C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (See e.g., Anderson and Young, Quantitative Filter

30 Hybridization, in Nucleic Acid Hybridization [1985]). Other references include more sophisticated computations that take structural as well as sequence characteristics into account for the calculation of T_m .

As used herein the term "stringency" is used in reference to the conditions of temperature, ionic strength, and the presence of other compounds such as organic solvents, under which nucleic acid hybridizations are conducted. With "high stringency" conditions, nucleic acid base pairing will occur only between nucleic acid fragments that have a high frequency of complementary base sequences. Thus, conditions of "weak" or "low" stringency are often required with nucleic acids that are derived from organisms that are genetically diverse, as the frequency of complementary sequences is usually less.

"Amplification" is a special case of nucleic acid replication involving template specificity. It is to be contrasted with non-specific template replication (i.e., replication that is template-dependent but not dependent on a specific template). Template specificity is here distinguished from fidelity of replication (i.e., synthesis of the proper polynucleotide sequence) and nucleotide (ribo- or deoxyribo-) specificity. Template specificity is frequently described in terms of "target" specificity. Target sequences are "targets" in the sense that they are sought to be sorted out from other nucleic acid. Amplification techniques have been designed primarily for this sorting out.

Template specificity is achieved in most amplification techniques by the choice of enzyme. Amplification enzymes are enzymes that, under conditions they are used, will process only specific sequences of nucleic acid in a heterogeneous mixture of nucleic acid. For example, in the case of Q β replicase, MDV-1 RNA is the specific template for the replicase (D. L. Kacian et al., Proc. Natl. Acad. Sci. USA 69:3038 [1972]). Other nucleic acid will not be replicated by this amplification enzyme. Similarly, in the case of T7 RNA polymerase, this amplification enzyme has a stringent specificity for its own promoters (M. Chamberlin et al., Nature 228:227 [1970]). In the case of T4 DNA ligase, the enzyme will not ligate the two oligonucleotides or polynucleotides, where there is a mismatch between the oligonucleotide or polynucleotide substrate and the template at the ligation junction (D. Y. Wu and R. B. Wallace, Genomics 4:560 [1989]).

Finally, Taq and Pfu polymerases, by virtue of their ability to function at high temperature, are found to display high specificity for the sequences bounded and thus defined by the primers; the high temperature results in thermodynamic conditions that favor primer hybridization with the target sequences and not
5 hybridization with non-target sequences (H. A. Erlich (ed.), PCR Technology, Stockton Press [1989]).

As used herein, the term "amplifiable nucleic acid" is used in reference to nucleic acids which may be amplified by any amplification method. It is
10 contemplated that "amplifiable nucleic acid" will usually comprise "sample template."

As used herein, the term "sample template" refers to nucleic acid originating from a sample that is analyzed for the presence of "target". In contrast, "background
15 template" is used in reference to nucleic acid other than sample template which may or may not be present in a sample. Background template is most often inadvertent. It may be the result of carryover, or it may be due to the presence of nucleic acid contaminants sought to be purified away from the sample. For example, nucleic acids from organisms other than those to be detected may be present as background
20 in a test sample.

As used herein, the term "primer" refers to an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, that is capable of acting as a point of initiation of synthesis when placed under conditions
25 in which synthesis of a primer extension product that is complementary to a nucleic acid strand is induced, (i.e., in the presence of nucleotides and an inducing agent such as DNA polymerase and at a suitable temperature and pH). The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to
30 separate its strands before being used to prepare extension products. Preferably, the primer is an oligodeoxyribonucleotide. The primer must be sufficiently long to prime the synthesis of extension products in the presence of the inducing agent. The

exact lengths of the primers will depend on many factors, including temperature, source of primer and the use of the method.

As used herein, the term "probe" refers to an oligonucleotide (i.e., a sequence of nucleotides), whether occurring naturally as in a purified restriction digest or produced synthetically, recombinantly or by PCR amplification, that is capable of hybridizing to another oligonucleotide of interest. A probe may be single-stranded or double-stranded. Probes are useful in the detection, identification and isolation of particular gene sequences. It is contemplated that any probe used in the present invention will be labelled with any "reporter molecule," so that is detectable in any detection system, including, but not limited to enzyme (e.g., ELISA, as well as enzyme-based histochemical assays), fluorescent, radioactive, and luminescent systems. It is not intended that the present invention be limited to any particular detection system or label.

15

As used herein, the term "target," refers to the region of nucleic acid bounded by the primers. Thus, the "target" is sought to be sorted out from other nucleic acid sequences. A "segment" is defined as a region of nucleic acid within the target sequence.

20

As used herein, the term "polymerase chain reaction" ("PCR") refers to the method of K. B. Mullis U.S. Pat. Nos. 4,683,195 4,683,202, and 4,965,188, hereby incorporated by reference, which describe a method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification. This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The

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steps of denaturation, primer annealing and polymerase extension can be repeated many times (i.e., denaturation, annealing and extension constitute one "cycle"; there can be numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified".

With PCR, it is possible to amplify a single copy of a specific target sequence in genomic DNA to a level detectable by several different methodologies (e.g., hybridization with a labeled probe; incorporation of biotinylated primers followed by avidin-enzyme conjugate detection; incorporation of ³²P-labeled deoxynucleotide triphosphates, such as dCTP or dATP, into the amplified segment). In addition to genomic DNA, any oligonucleotide or polynucleotide sequence can be amplified with the appropriate set of primer molecules. In particular, the amplified segments created by the PCR process are, themselves, efficient templates for subsequent PCR amplifications.

As used herein, the terms "PCR product," "PCR fragment," and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete. These terms encompass the case where there has been amplification of one or more segments of one or more target sequences.

As used herein, the term "amplification reagents" refers to those reagents (deoxyribonucleotide triphosphates, buffer, etc.), needed for amplification except for primers, nucleic acid template and the amplification enzyme. Typically, amplification reagents along with other reaction components are placed and contained in a reaction vessel (test tube, microwell, etc.).

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

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As used herein, the term "antisense" is used in reference to DNA or RNA sequences that are complementary to a specific DNA or RNA sequence (e.g., mRNA). Included within this definition are antisense RNA ("asRNA") molecules involved in gene regulation by bacteria. Antisense RNA may be produced by any method, including synthesis by splicing the gene(s) of interest in a reverse orientation to a viral promoter which permits the synthesis of a coding strand. Once introduced into an embryo, this transcribed strand combines with natural mRNA produced by the embryo to form duplexes. These duplexes then block either the further transcription of the mRNA or its translation. In this manner, mutant phenotypes may be generated. The term "antisense strand" is used in reference to a nucleic acid strand that is complementary to the "sense" strand. The designation (-) (i.e., "negative") is sometimes used in reference to the antisense strand, with the designation (+) sometimes used in reference to the sense (i.e., "positive") strand.

The terms "in operable combination," "in operable order," and "operably linked" as used herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of amino acid sequences in such a manner so that a functional protein is produced.

The term "isolated" when used in relation to a nucleic acid, as in "an isolated oligonucleotide" or "isolated polynucleotide" refers to a nucleic acid sequence that is identified and separated from at least one contaminant nucleic acid with which it is ordinarily associated in its natural source. Isolated nucleic acid is such present in a form or setting that is different from that in which it is found in nature. In contrast, non-isolated nucleic acids as nucleic acids such as DNA and RNA found in the state

they exist in nature. For example, a given DNA sequence (e.g., a gene) is found on the host cell chromosome in proximity to neighboring genes; RNA sequences, such as a specific mRNA sequence encoding a specific protein, are found in the cell as a mixture with numerous other mRNAs that encode a multitude of proteins. However, 5 isolated nucleic acid encoding a given protein includes, by way of example, such nucleic acid in cells ordinarily expressing the given protein where the nucleic acid is in a chromosomal location different from that of natural cells, or is otherwise flanked by a different nucleic acid sequence than that found in nature. The isolated nucleic acid, oligonucleotide, or polynucleotide may be present in single-stranded or 10 double-stranded form. When an isolated nucleic acid, oligonucleotide or polynucleotide is to be utilized to express a protein, the oligonucleotide or polynucleotide will contain at a minimum the sense or coding strand (i.e., the oligonucleotide or polynucleotide may be single-stranded), but may contain both the sense and anti-sense strands (i.e., the oligonucleotide or polynucleotide may be 15 double-stranded).

The term "transgene" as used herein refers to a foreign gene that is placed into an organism by, for example, introducing the foreign gene into newly fertilized eggs or early embryos. The term "foreign gene" refers to any nucleic acid (e.g., 20 gene sequence) that is introduced into the genome of an animal by experimental manipulations and may include gene sequences found in that animal so long as the introduced gene does not reside in the same location as does the naturally-occurring gene.

25 Embryonal cells at various developmental stages can be used to introduce transgenes for the production of transgenic animals. Different methods are used depending on the stage of development of the embryonal cell. The zygote is a preferred target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible 30 injection of 1-2 picoliters (pl) of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host genome before the first cleavage (Brinster et al., Proc.

Natl. Acad. Sci. USA 82:4438-4442 [1985]). As a consequence, all cells of the transgenic non-human animal will carry the incorporated transgene. This will, in general, also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Micro-injection of zygotes is the preferred method for incorporating transgenes in practicing the invention. U.S. Pat. No. 4,873,191 describes a method for the micro-injection of zygotes; the disclosure of this patent is incorporated herein in its entirety.

10 Retroviral infection can also be used to introduce transgenes into an animal. The developing embryo can be cultured in vitro to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Janenich, Proc. Natl. Acad. Sci. USA 73:1260-1264 [1976]). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (Hogan et al., in
15 Manipulating the Mouse Embryo, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. [1986]). The viral vector system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (D. Jahner et al., Proc. Natl. Acad. Sci. USA 82:6927-693 [1985]). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-
20 producing cells (Van der Putten, supra; Stewart, et al, EMBO J. 6:383-388 [1987]). Alternatively, infection can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (D. Jahner et al, Nature 298:623-628 [1982]). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of cells that form the transgenic animal. Further, the founder
25 may contain various retroviral insertions of the transgene at different positions in the genome that generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germline, albeit with low efficiency, by intrauterine retroviral infection of the midgestation embryo (Jahner et al., supra [1982]). Additional means of using retroviruses or retroviral vectors to create transgenic
30 animals known to the art involves the micro-injection of retroviral particles or mitomycin C-treated cells producing retrovirus into the perivitelline space of

fertilized eggs or early embryos (PCT International Application WO 90/08832 [1990], and Haskell and Bowen, *Mol. Reprod. Dev.*, 40:386 [1995]).

5 A third type of target cell for transgene introduction is the embryonal stem (ES) cell. ES cells are obtained by culturing pre-implantation embryos in vitro under appropriate conditions (Evans et al., *Nature* 292:154-156 [1981]; Bradley et al., *Nature* 309:255-258 [1984]; Gossler et al, *Proc. Acad. Sci. USA* 83:9065-9069 [1986]; and Robertson et al., *Nature* 322:445-448 [1986]). Transgenes can be efficiently introduced into the ES cells by DNA transfection by a variety of methods
10 known to the art including calcium phosphate co-precipitation, protoplast or spheroplast fusion, lipofection and DEAE-dextran-mediated transfection. Transgenes may also be introduced into ES cells by retrovirus-mediated transduction or by micro-injection. Such transfected ES cells can thereafter colonize an embryo following their introduction into the blastocoel of a blastocyst-stage embryo and
15 contribute to the germ line of the resulting chimeric animal (for review, See, Jaenisch, *Science* 240:1468-1474 [1988]). Prior to the introduction of transfected ES cells into the blastocoel, the transfected ES cells may be subjected to various selection protocols to enrich for ES cells that have integrated the transgene assuming that the transgene provides a means for such selection. Alternatively, the
20 polymerase chain reaction may be used to screen for ES cells that have integrated the transgene. This technique obviates the need for growth of the transfected ES cells under appropriate selective conditions prior to transfer into the blastocoel.

The terms "overexpression" and "overexpressing" and grammatical
25 equivalents, are used in reference to levels of mRNA to indicate a level of expression approximately 3-fold higher than that typically observed in a given tissue in a control or non-transgenic animal. Levels of mRNA are measured using any of a number of techniques known to those skilled in the art including, but not limited to Northern blot analysis. Appropriate controls are included on the Northern blot to
30 control for differences in the amount of RNA loaded from each tissue analyzed (e.g., the amount of 28S rRNA, an abundant RNA transcript present at essentially the same amount in all tissues, present in each sample can be used as a means of

normalizing or standardizing the mRNA-specific signal observed on Northern blots). The amount of mRNA present in the band corresponding in size to the correctly spliced transgene RNA is quantified; other minor species of RNA which hybridize to the transgene probe are not considered in the quantification of the expression of the transgenic mRNA.

The term "transfection" as used herein refers to the introduction of foreign DNA into eukaryotic cells. Transfection may be accomplished by a variety of means known to the art including calcium phosphate-DNA co-precipitation, DEAE-dextran-mediated transfection, polybrene-mediated transfection, electroporation, microinjection, liposome fusion, lipofection, protoplast fusion, retroviral infection, and biolistics.

The term "stable transfection" or "stably transfected" refers to the introduction and integration of foreign DNA into the genome of the transfected cell. The term "stable transfectant" refers to a cell which has stably integrated foreign DNA into the genomic DNA.

The term "transient transfection" or "transiently transfected" refers to the introduction of foreign DNA into a cell where the foreign DNA fails to integrate into the genome of the transfected cell. The foreign DNA persists in the nucleus of the transfected cell for several days. During this time the foreign DNA is subject to the regulatory controls that govern the expression of endogenous genes in the chromosomes. The term "transient transfectant" refers to cells which have taken up foreign DNA but have failed to integrate this DNA.

The term "calcium phosphate co-precipitation" refers to a technique for the introduction of nucleic acids into a cell. The uptake of nucleic acids by cells is enhanced when the nucleic acid is presented as a calcium phosphate-nucleic acid co-precipitate. The original technique of Graham and van der Eb (Graham and van der Eb, *Virology*, 52:456 [1973]), has been modified by several groups to optimize

conditions for particular types of cells. The art is well aware of these numerous modifications.

As used herein, the term "selectable marker" refers to the use of a gene that
5 encodes an enzymatic activity that confers the ability to grow in medium lacking
what would otherwise be an essential nutrient (e.g. the HIS3 gene in yeast cells); in
addition, a selectable marker may confer resistance to an antibiotic or drug upon the
cell in which the selectable marker is expressed. Selectable markers may be
"dominant"; a dominant selectable marker encodes an enzymatic activity that can be
10 detected in any eukaryotic cell line. Examples of dominant selectable markers
include the bacterial aminoglycoside 3' phosphotransferase gene (also referred to as
the neo gene) that confers resistance to the drug G418 in mammalian cells, the
bacterial hygromycin G phosphotransferase (hyg) gene that confers resistance to the
antibiotic hygromycin and the bacterial xanthine-guanine phosphoribosyl transferase
15 gene (also referred to as the gpt gene) that confers the ability to grow in the presence
of mycophenolic acid. Other selectable markers are not dominant in that their use
must be in conjunction with a cell line that lacks the relevant enzyme activity.
Examples of non-dominant selectable markers include the thymidine kinase (tk)
gene that is used in conjunction with tk- cell lines, the CAD gene which is used in
20 conjunction with CAD-deficient cells and the mammalian hypoxanthine-guanine
phosphoribosyl transferase (hpert) gene which is used in conjunction with hpert- cell
lines. A review of the use of selectable markers in mammalian cell lines is provided
in Sambrook, J. et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold
Spring Harbor Laboratory Press, New York (1989) pp.16.9-16.15.

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As used herein, the term "purified" or "to purify" refers to the removal of
contaminants from a sample. For example, antibodies are purified by removal of
contaminating non-immunoglobulin proteins; they are also purified by the removal
of immunoglobulin that does not bind to the target molecule. The removal of non-
30 immunoglobulin proteins and/or the removal of immunoglobulins that do not bind to
the target molecule results in an increase in the percent of target-reactive
immunoglobulins in the sample. In another example, recombinant polypeptides are

expressed in bacterial host cells and the polypeptides are purified by the removal of host cell proteins; the percent of recombinant polypeptides is thereby increased in the sample.

5 The term "Western blot" refers to the analysis of protein(s) (or polypeptides) immobilized onto a support such as nitrocellulose or a membrane. The proteins are run on acrylamide gels to separate the proteins, followed by transfer of the protein from the gel to a solid support, such as nitrocellulose or a nylon membrane. The immobilized proteins are then exposed to antibodies with reactivity against an
10 antigen of interest. The binding of the antibodies may be detected by various methods, including the use of radiolabelled antibodies.

 The term "antigenic determinant" as used herein refers to that portion of an antigen that makes contact with a particular antibody (i.e., an epitope). When a
15 protein or fragment of a protein is used to immunize a host animal, numerous regions of the protein may induce the production of antibodies which bind specifically to a given region or three-dimensional structure on the protein; these regions or structures are referred to as antigenic determinants. An antigenic determinant may compete with the intact antigen (i.e., the "immunogen" used to
20 elicit the immune response) for binding to an antibody.

 The terms "specific binding" or "specifically binding" when used in reference to the interaction of an antibody and a protein or peptide means that the interaction is dependent upon the presence of a particular structure (i.e., the
25 antigenic determinant or epitope) on the protein; in other words the antibody is recognizing and binding to a specific protein structure rather than to proteins in general. For example, if an antibody is specific for epitope "A," the presence of a protein containing epitope A (or free, unlabelled A) in a reaction containing labeled "A" and the antibody will reduce the amount of labeled A bound to the antibody.

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 As used herein, the term "cell culture" refers to any in vitro culture of cells. Included within this term are continuous cell lines (e.g., with an immortal

phenotype), primary cell cultures, finite cell lines (e.g., non-transformed cells), and any other cell population maintained in vitro.

As used, the term "eukaryote" refers to organisms distinguishable from "prokaryotes." It is intended that the term encompass all organisms with cells that exhibit the usual characteristics of eukaryotes, such as the presence of a true nucleus bounded by a nuclear membrane, within which lie the chromosomes, the presence of membrane-bound organelles, and other characteristics commonly observed in eukaryotic organisms. Thus, the term includes, but is not limited to such organisms as fungi, protozoa, and animals (e.g., humans).

As used herein, the term "in vitro" refers to an artificial environment and to processes or reactions that occur within an artificial environment. In vitro environments consist of, but are not limited to, test tubes and cell culture. The term "in vivo" refers to the natural environment (e.g., an animal or a cell) and to processes or reaction that occur within a natural environment.

The term "test compound" refers to any chemical entity, pharmaceutical, drug, and the like that are used to treat or prevent a disease, illness, sickness, or disorder of bodily function. Test compounds comprise both known and potential therapeutic compounds. A test compound can be determined to be therapeutic by screening using the screening methods of the present invention. A "known therapeutic compound" refers to a therapeutic compound that has been shown (e.g., through animal trials or prior experience with administration to humans) to be effective in such treatment or prevention.

As used herein, the term "sample" is used in its broadest sense. In one sense it can refer to drugs and therapeutic compounds. In another sense, it is meant to include a specimen or culture obtained from any source, as well as biological and environmental samples. Biological samples may be obtained from animals (including humans) and encompass fluids, solids, tissues, and gases. Biological samples include blood products, such as plasma, serum and the like. Environmental

samples include environmental material such as surface matter, soil, water, and industrial samples. These examples are not to be construed as limiting the sample types applicable to the present invention.

5 **General Description of the Invention**

The present invention relates to methods and compositions for treating Parkinson's disease (PD). In particular, the present invention provides agents that regulate the production of proinflammatory and neurotoxic products involved in Parkinson's disease.

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As discussed above, the therapeutic treatment strategies that have been used against PD have targeted neurotransmitter replacement, or the preservation of normal brain structures, which potentially provide short-time relief, but do not prevent neuronal degeneration and death. In response to this need for more effective
15 treatments against PD, the present invention provides means to prevent neuronal degeneration and death through regulation of inflammatory processes.

The presence of inflammatory cytokines at elevated levels in the PD brain and the presence of a number of acute phase products has been reported. A
20 microglial activation has been demonstrated in the substantia nigra of PD patients, in human patients exposed to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), in an MPTP-induced mouse model of PD, and in 6-hydroxydopamine-induced parkinsonism rats. Activation of microglia becomes apparent by proliferation, recruitment to site of injury, and by morphological, immunohistochemical and
25 functional changes. Activated microglia is believed to contribute to neurodegeneration through the release of cytotoxic compounds including reactive oxygen intermediates, nitric oxide, proteases and pro-inflammatory cytokines. Prior to the present invention, the molecular mechanisms underlying these inflammatory processes have not been sufficiently characterized, and safe, preventative therapies
30 have not been developed to prevent the associated neuronal degeneration and death.

The compositions and methods of the present invention provide means to inhibit a diverse range of microglial response to PD. For example, the present invention provides agents that suppress a broad range of inflammatory responses (e.g., a broad range of inflammatory responses in monocytes and microglia). These agents (i.e., PPAR γ agonists) are shown to interact with the transcription factor PPAR γ . The present invention also demonstrates that PPAR γ agonists block the expression of cyclooxygenase-2 (COX-2) and the cytokines TNF- α and IL-6, and inhibit the secretion of neurotoxic products. Prior to the present invention, the therapeutic effects of PPAR γ and PPAR γ effectors in inflammatory disease were unexplored.

Thus, the present invention provides methods and compositions for attenuating the progressive neurodegenerative processes in Parkinson's disease. However, it is not intended that the present invention be limited to any particular mechanism. Indeed, an understanding of the mechanisms is not necessary in order to practice the present invention.

Detailed Description of the Invention

The present invention comprises methods and compositions for treating Parkinson's disease (PD). In particular, the present invention provides agents that regulate the production of proinflammatory and neurotoxic products involved in PD. The therapeutic agents of the present invention comprise PPAR γ ligands (e.g., PPAR γ agonists). Although it is not necessary to understand the mechanisms in order to practice the present invention, and it is not intended that the present invention be so limited, it is contemplated that the therapeutic agents of the present invention regulate the production of proinflammatory and neurotoxic through the alteration PPAR γ activity and subsequent regulation of gene expression by PPAR γ .

The PPARs are lipid-activated DNA binding proteins that are structurally related to the steroid and retinoic acid receptor families (Lemberger et al., Annu. Rev. Cell Dev. Biol. 12:335 [1996]). The activated form of the receptor binds to sequence-specific promoter elements, termed PPREs, and transcriptionally regulate gene

expression (Ricote et al., Nature 391:79 [1998]). There are three PPAR isoforms (PPAR α , γ , and Δ) which are differentially expressed. The natural ligands for this receptor family are fatty acids and lipid metabolites, with each PPAR family member displaying a distinct pattern of ligand specificity.

5

I. Therapeutic Agents of the Present Invention

The present invention demonstrates that agents regulating PPAR γ (e.g., PPAR γ agonists) provide therapeutic compositions that regulate the production of proinflammatory and neurotoxic products involved in Parkinson's disease. Such agents include, but are not limited to, prostaglandin J₂ (PGJ₂) and analogs thereof (e.g., Δ^{12} -prostaglandin J₂ and 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂), members of the prostaglandin D₂ family of compounds, docosahexaenoic acid (DHA), and thiazolidinediones (e.g., ciglitazone, troglitazone, pioglitazone, and rosiglitazone). In addition, such agents include, but are not limited to, L-tyrosine-based compounds, farglitazar, GW7845, indole-derived compounds, indole 5-carboxylic acid derivatives and 2,3-disubstituted indole 5-phenylacetic acid derivatives. It is significant that most of the PPAR γ agonists exhibit substantial bioavailability following oral administration and have little or no toxicity associated with their use (See e.g., Saltiel and Olefsky, Diabetes 45:1661 [1996]; Wang et al, Br. J. Pharmacol. 122:1405 [1997]; and Oakes et al, Metabolism 46:935 [1997]). The present invention contemplates that any known or future identified PPAR γ agonist will find use with the present invention.

Compounds useful for practicing the present invention, and methods of making these compounds are disclosed in WO 91/07107; WO 92/02520; WO 94/01433; WO 89/08651; WO 96/33724; WO 97/31907; U.S. Pat. Nos. 4,287,200; 4,340,605; 4,438,141; 4,444,779; 4,461,902; 4,572,912; 4,687,777; 4,703,052; 4,725,610; 4,873,255; 4,897,393; 4,897,405; 4,918,091; 4,948,900; 5,002,953; 5,061,717; 5,120,754; 5,132,317; 5,194,443; 5,223,522; 5,232,925; 5,260,445; 5,814,647; 5,902,726; 5,994,554; 6,294,580; 6,306,854; 6,498,174; 6,506,781; 6,541,492; ; and U.S. Applications 20030130306, 20030134885, 20030109579, 20030109560, 20030088103, 20030087902, 20030096846, 20030092697, 20030087935,

20030082631, 20030078288, 20030073862, 20030055265, 20030045553, 20020169192, 20020165282, 20020160997, 20020128260, 20020103188, 20020082292, 20030092736, 20030069275, 20020151569, and 20030064935, as well as Mital et al., CRIPS 2002, 3(2), 5-8, Liu et al., Biorg. Med. Chem. Lett. 11 (2001) 3111-3113, and Henke et al. Biorg. Med. Chem. Lett. 9:3329-3334, 1999. The disclosures of these publications are incorporated herein by reference in their entireties, especially with respect to the PPAR γ agonists disclosed therein, which may be employed in the methods described herein.

- 10 As agents having the aforementioned effects the compounds of the following formulas are useful in treating individuals. Accordingly, in some embodiments of the present invention, the therapeutic agents comprise compounds of Formula I in FIG. 8, wherein R₁ and R₂ are the same or different, and each represents a hydrogen atom or a C₁-C₅ alkyl group; R₃ represents a hydrogen atom, a C₁-C₆ aliphatic acyl group, an alicyclic acyl group, an aromatic acyl group, a heterocyclic acyl group, an araliphatic acyl group, a (C₁-C₆ alkoxy)carbonyl group, or an aralkyloxycarbonyl group; R₄ and R₅ are the same or different, and each represents a hydrogen atom, a C₁-C₅ alkyl group or a C₁-C₅ alkoxy group, or R₄ and R₅ together represent a C₁-C₅ alkylendioxy group; n is 1, 2, or 3; W represents the --CH₂-- , >CO, or CH--OR₆ group (in which R₆ represents any one of the atoms or groups defined for R₃ and may be the same as or different, from R₃); and Y and Z are the same or different and each represents an oxygen atom or an imino (=NH) group; and pharmaceutically acceptable salts thereof.
- 25 In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula II in FIG. 8, wherein R₁₁ is a substituted or unsubstituted alkyl, alkoxy, cycloalkyl, phenylalkyl, phenyl, aromatic acyl group, a 5- or 6-membered heterocyclic group including 1 or 2 heteroatoms selected from the group consisting of nitrogen, oxygen, and sulfur, or a group of the formula indicated in FIG. 8 (i.e., the group labeled "Possible Formula Used for R₁₁ ") wherein R₁₃ and R₁₄ are the same or different and each is lower alkyl (alternately, R₁₃ and R₁₄ are combined to each other either directly or as interrupted by a heteroatom comprising

nitrogen, oxygen, and sulfur to form a 5- or 6-membered ring); and wherein L^1 and L^2 are the same or different and each is hydrogen or lower alkyl or L^1 and L^2 are combined to form an alkylene group; or a pharmaceutically acceptable salt thereof.

- 5 In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula III in FIG. 8, wherein R_{15} and R_{16} are independently hydrogen, lower alkyl containing 1 to 6 carbon atoms, alkoxy containing 1 to 6 carbon atoms, halogen, ethynyl, nitrile, methylthio, trifluoromethyl, vinyl, nitro, or halogen substituted benzyloxy; n is 0 to 4; or a pharmaceutically acceptable salt
10 thereof.

- In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula IV in FIG. 8, wherein the dotted line represents a bond or no bond; V is $-H=CH-$, $-N=CH-$, $-CH=N-$, or S ; D is CH_2 , $CHOH$, CO ,
15 $C=NOR_{17}$, or $CH=CH$; X is S , O , NR_{18} , $-CH=N$, or $-N=CH$; Y is CH or N ; Z is hydrogen, (C_1-C_7) alkyl, (C_1-C_7) cycloalkyl, phenyl, naphthyl, pyridyl, furyl, thienyl, or phenyl mono- or di-substituted with the same or different groups which are (C_1-C_3) alkyl, trifluoromethyl, (C_1-C_3) alkoxy, fluoro, chloro, or bromo; Z^1 is hydrogen or (C_1-C_3) alkyl; R_{17} and R_{18} are each independently hydrogen or methyl;
20 and n is 1, 2, or 3; the pharmaceutically acceptable cationic salts thereof; and the pharmaceutically acceptable acid addition salts thereof when the compound contains a basic nitrogen.

- In some embodiments of the present invention, the therapeutic agents comprise
25 compounds of Formula V in FIG. 8, wherein the dotted line represents a bond or no bond; A and B are each independently CH or N , with the proviso that when A or B is N , the other is CH ; X^1 is S , SO , SO_2 , CH_2 , $CHOH$, or CO ; n is 0 or 1; Y_1 is CHR^{20} or R^{21} , with the proviso that when n is 1 and Y_1 is NR^{21} , X^1 is SO_2 or CO ; Z^2 is CHR^{22} , CH_2CH_2 , cyclic C_2H_2O , $CH=CH$, OCH_2 , SCH_2 , $SOCH_2$, or SO_2CH_2 ;
30 R^{19} , R^{20} , R^{21} , and R^{22} are each independently hydrogen or methyl; and X^2 and X^3 are each independently hydrogen, methyl, trifluoromethyl, phenyl, benzyl, hydroxy, methoxy, phenoxy, benzyloxy, bromo, chloro, or fluoro; a pharmaceutically

acceptable cationic salt thereof; or a pharmaceutically acceptable acid addition salt thereof when A or B is N.

5 In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula VI in FIG. 8, or a pharmaceutically acceptable salt thereof, wherein R²³ is alkyl of 1 to 6 carbon atoms, cycloalkyl of 3 to 7 carbon atoms, phenyl or mono- or di-substituted phenyl wherein said substituents are independently alkyl of 1 to 6 carbon atoms, alkoxy of 1 to 3 carbon atoms, halogen, or trifluoromethyl.

10

In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula VII in FIG. 8, or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein: A² represents an alkyl group, a substituted or unsubstituted aryl group, or an aralkyl group wherein the alkylene or the aryl moiety may be substituted or unsubstituted; A³ represents a benzene ring having in total up to 3 optional substituents; R²⁴ represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group wherein the alkyl or the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group; or A² together with R²⁴ represents substituted or unsubstituted C₂₋₃ polymethylene group, optional substituents for the polymethylene group being selected from alkyl or aryl or adjacent substituents together with the methylene carbon atoms to which they are attached form a substituted or unsubstituted phenylene group; R²⁵ and R²⁶ each represent hydrogen, or R²⁵ and R²⁶ together represent a bond; X⁴ represents O or S; 15 20 25 and n represents an integer in the range from 2 to 6.

In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula VIII in FIG. 8, or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein: R²⁷ and R²⁸ each independently represent an alkyl group, a substituted or unsubstituted aryl group, or an aralkyl group being substituted or unsubstituted in the aryl or alkyl moiety; or R²⁷ together with R²⁸ represents a 30

linking group, the linking group consisting or an optionally substituted methylene group or an O or S atom, optional substituents for the methylene groups including alky-, aryl, or aralkyl, or substituents of adjacent methylene groups together with the carbon atoms to which they are attached form a substituted or unsubstituted phenylene group; R²⁹ and R³⁰ each represent hydrogen, or R²⁹ and R³⁰ together represent a bond; A⁴ represents a benzene ring having in total up to 3 optional substituents; X⁵ represents O or S; and n represents an integer in the range of 2 to 6.

In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula IX in FIG. 8, or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein: A⁵ represents a substituted or unsubstituted aromatic heterocyclyl group; A⁶ represents a benzene ring having in total up to 5 substituents; X⁶ represents O, S, or NR³² wherein R³² represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group; Y² represents O or S; R³¹ represents an alkyl, aralkyl, or aryl group; and n represents an integer in the range from 2 to 6. Suitable aromatic heterocyclyl groups include substituted or unsubstituted, single or fused ring aromatic heterocyclyl groups comprising up to 4 hetero atoms in each ring selected from oxygen, sulfur, or nitrogen. Favored aromatic heterocyclyl groups include substituted or unsubstituted single ring aromatic heterocyclyl groups having 4 to 7 ring atoms, preferably 5 or 6 ring atoms. In particular, the aromatic heterocyclyl group comprises 1, 2, or 3 heteroatoms, especially 1 or 2, selected from oxygen, sulfur, or nitrogen. Suitable values for A⁵ when it represents a 5-membered aromatic heterocyclyl group include thiazolyl and oxazolyl, especially oxazolyl. Suitable values for A⁶ when it represents a 6-membered aromatic heterocyclyl group include pyridyl or pyrimidinyl. Suitable R³¹ represents an alkyl group, in particular a C₁₋₆ alkyl group (e.g., a methyl group). Preferably, A⁵ represents a moiety of formula (a), (b), or (c), in FIG. 8, under Formula IX: formula (a), (b), and (c) wherein: R³³ and R³⁴ each independently represents a hydrogen atom, an alkyl group, or a substituted or unsubstituted aryl group or when R³³ and R³⁴ are each attached to adjacent carbon atoms, then R³³ and

R³⁴ together with the carbon atoms to which they are attached form a benzene ring wherein each carbon atom represented by R³³ and R³⁴ together may be substituted or unsubstituted; and in the moiety of Formula (a), X⁷ represents oxygen or sulphur. In one preferred embodiment of the present invention, R³³ and R³⁴ together present a moiety of Formula (d) in FIG. 8, under Formula IX: wherein R³⁵ and R³⁶ each independently represent hydrogen, halogen, substituted or unsubstituted alkyl, or alkoxy.

In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula X in FIG. 8, or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein: A⁷ represents a substituted or unsubstituted aryl group; A⁸ represents a benzene ring having in total up to 5 substituents; X⁸ represents O, S, or NR⁹, wherein R⁹ represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group; Y³ represents O or S; R³⁷ represents hydrogen; R³⁸ represents hydrogen or an alkyl, aralkyl, or aryl group or R³⁷ together with R³⁸ represents a bond; and n represents an integer in the range from 2 to 6.

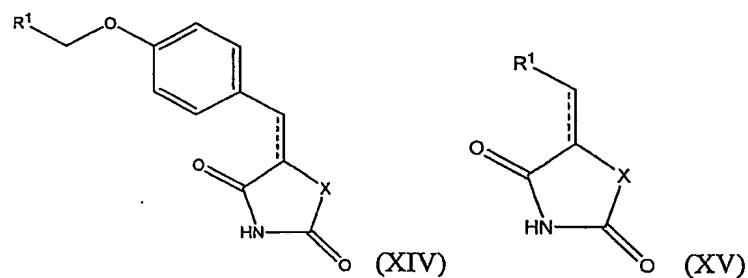
In some embodiments of the present invention, the therapeutic agents comprise compounds of Formula XI in FIG. 8, or a tautomeric form thereof and/or a pharmaceutically acceptable salt thereof, and/or a pharmaceutically acceptable solvate thereof, wherein: A¹ represents a substituted or unsubstituted aromatic heterocyclyl group; R¹ represents a hydrogen atom, an alkyl group, an acyl group, an aralkyl group, wherein the aryl moiety may be substituted or unsubstituted, or a substituted or unsubstituted aryl group; A² represents a benzene ring having in total up to 5 substituents; and n represents an integer in the range of from 2 to 6. Suitable aromatic heterocyclyl groups include substituted or unsubstituted, single or fused ring aromatic heterocyclyl groups comprising up to 4 hetero atoms in each ring selected from oxygen, sulfur, or nitrogen. Favored aromatic heterocyclyl groups include substituted or unsubstituted single ring aromatic heterocyclyl groups having 4 to 7 ring atoms, preferably 5 or 6 ring atoms. In particular, the aromatic

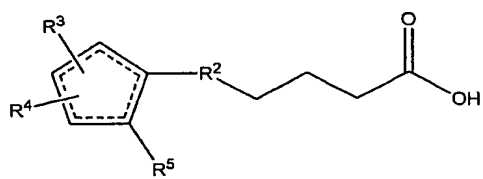
heterocyclyl group comprises 1, 2, or 3 heteroatoms, especially 1 or 2, selected from oxygen, sulfur, or nitrogen. Suitable values for A¹ when it represents a 5-membered aromatic heterocyclyl group include thiazolyl and oxazolyl, especially oxazolyl. Suitable values for A¹ when it represents a 6-membered aromatic heterocyclyl group include pyridyl or pyrimidinyl.

In some embodiments of the present invention, the therapeutic agent comprises a compound of Formulas XII and XIII in FIG. 8, or pharmaceutically acceptable salts thereof wherein the dotted line represents a bond or no bond; R is cycloalkyl of three to seven carbon atoms, naphthyl, thienyl, furyl, phenyl, or substituted phenyl wherein the substituent is alkyl of one to three carbon atoms, alkoxy of one to three carbon atoms, trifluoromethyl, chloro, fluoro, or bis(trifluoromethyl); R¹ is alkyl of one to three carbon atoms; X is O or C=O; A is O or S; and B is N or CH.

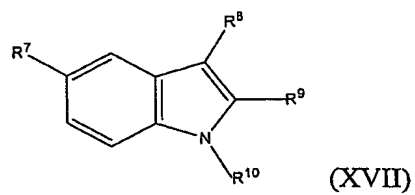
Some embodiments of the present invention include the use of the compounds of Formulas I through XIII for the treatment of Parkinson's disease. These compounds are herein referred to as thiazolidine derivatives. Where appropriate, the specific names of thiazolidine derivatives may be used including: troglitazone, ciglitazone, pioglitazone, and rosiglitazone.

In certain embodiments, the therapeutic agent comprises an activator of PPAR γ as described in U.S. Patent 5,994,554, e.g., having a structure selected from the group consisting of formula (XIV)-(XXVI):

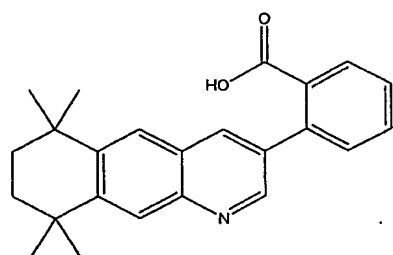




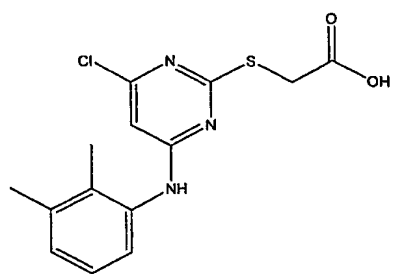
(XVI)



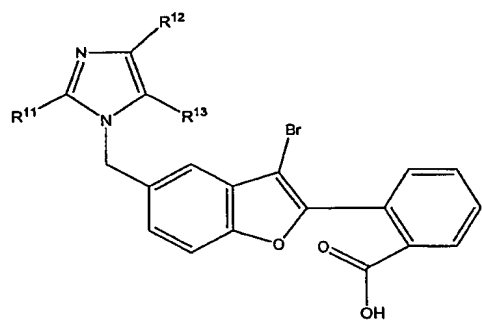
(XVII)



(XVIII)

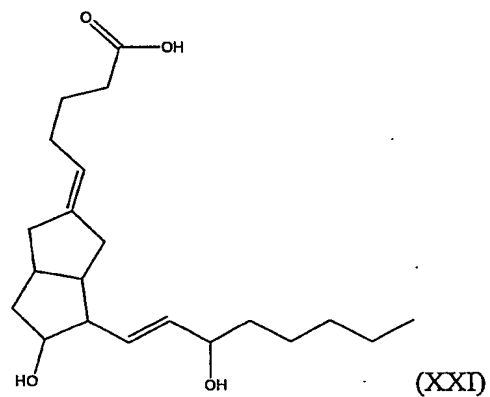


(XIX)

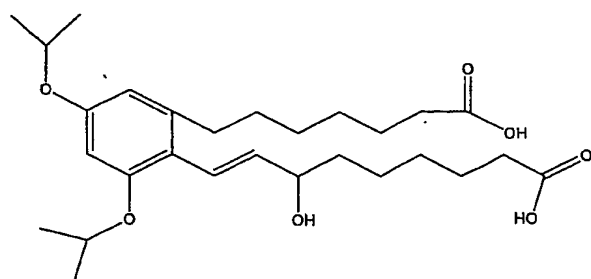


(XX)

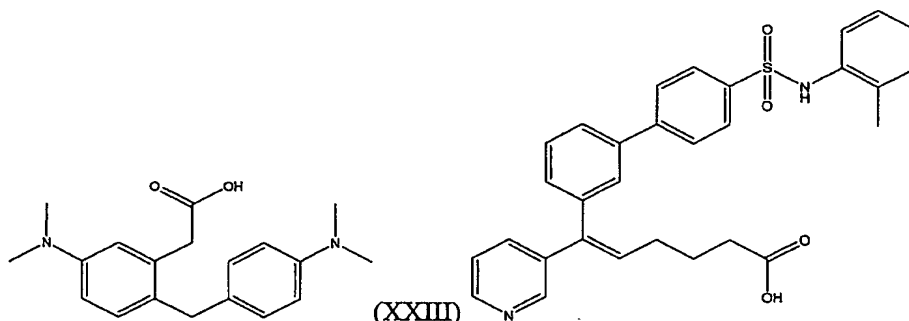
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(XXI)

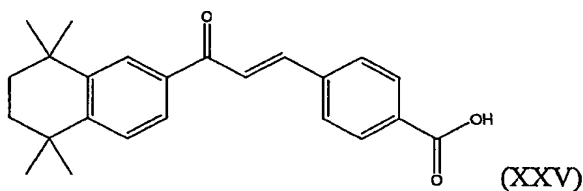


(XXII)



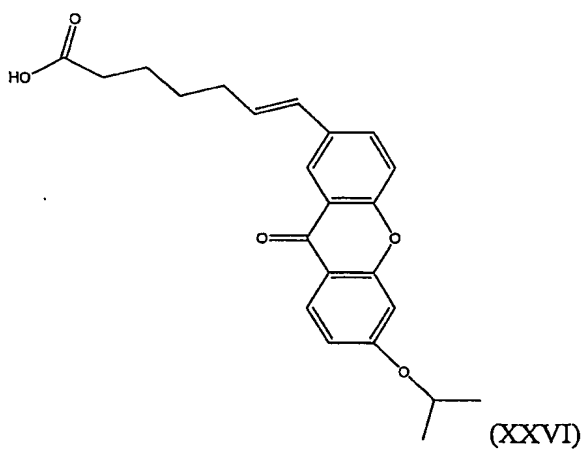
(XXIII)

(XXIV)



(XXV)

5



(XXVI)

wherein: R^1 is selected from the group consisting of hydrogen, C_{1-8} alkyl, amino C_{1-8} alkyl, C_{1-8} alkylamino C_{1-8} alkyl, heteroaryl amino C_{1-6} alkyl, (heteroaryl)($C_{sub.1-8}$)

alkyl)aminoC₁₋₆ alkyl, (C₁₋₈ cycloalkyl)C₁₋₈ alkyl, C₁₋₈ alkylheteroarylC₁₋₈ alkyl, 9- or 10-membered heterobicycle which is partially aromatic or substituted 9- or 10-membered heterobicycle which is partially aromatic;

5 X is selected from the group consisting of S, NH or O;

R² is selected from the group consisting of hydrogen, C₁₋₈alkyl or C₁₋₈alkenyl;

R³ and R⁴ are independently selected from the group consisting of hydrogen,
10 hydroxy, oxo, C₁₋₈alkyl, C₁₋₈alkoxy or amino;

R⁵ is selected from the group consisting of hydrogen, C₁₋₈alkyl, C₁₋₈alkenyl,
(carbonyl)alkenyl, (hydroxy)alkenyl, phenyl, C₁₋₈alkylR⁶, (hydroxy)C₁₋₈alkylR⁶, C₁₋₈alkylC₁₋₈cycloalkylR⁶, (hydroxy)C₁₋₈alkylC₁₋₈cycloalkylR⁶ or C₁₋₈cycloalkylthioR⁶;
15

R⁶ is selected from the group consisting of phenyl or phenyl substituted with
hydroxy, C₁₋₈alkyl or C₁₋₈alkoxy substituents;

R⁷ is selected from the group consisting of hydrogen, hydroxy, carboxy or
20 carboxyC₁₋₈alkyl;

R⁸ is selected from the group consisting of hydrogen, C₁₋₈alkyl, phenyl, phenylC₁₋₈alkyl, phenyl mono- or di-substituted with halo, hydroxy, and/or C₁₋₈alkoxy (e.g., methoxy) substituents or phenylC₁₋₈alkyl wherein the phenyl is mono- or
25 disubstituted with halo, hydroxy, and/or C₁₋₈alkoxy (e.g., methoxy) substituents;

R⁹ is selected from the group consisting of hydrogen, C₁₋₈alkyl, carboxyC₁₋₈alkenyl mono- or disubstituted with hydroxy, and/or C₁₋₈alkoxy (e.g., methoxy), phenyl or phenyl mono- or disubstituted with halo, hydroxy, and/or C₁₋₈alkoxy (e.g., methoxy)
30 substituents;

R¹⁰ is hydrogen or C₁₋₈alkyl;

R¹¹ is selected from the group consisting of hydrogen, C₁₋₈alkyl or cycloC₁₋₈alkylC₁₋₈alkyl;

- 5 R¹² is selected from the group consisting of hydrogen, halo or fluorinated C₁₋₈ alkyl;

R¹³ is selected from the group consisting of hydrogen, C₁₋₈alkoxycarbonyl or C₁₋₈alkoxycarbonylC₁₋₈alkylaminocarbonyl;

- 10 A dashed line (- - -) is none or one double bond between two of the carbon atoms;

Fluorinated alkyl in more detail is an alkyl wherein one or more of the hydrogen atoms is replaced by a fluorine atom;

- 15 heteroaryl in more detail is a 5, 6 or 7 membered aromatic ring optionally interrupted by 1, 2, 3 or 4 N, S, or O heteroatoms, with the proviso that any two O or S atoms are not bonded to each other;

- substituted heteroaryl in more detail is a 9- or 10-membered heterobicycle mono-,
20 di-, or trisubstituted independently with hydroxy, oxo, C₁₋₆ alkyl, C₁₋₆ alkoxy or amino substituents;

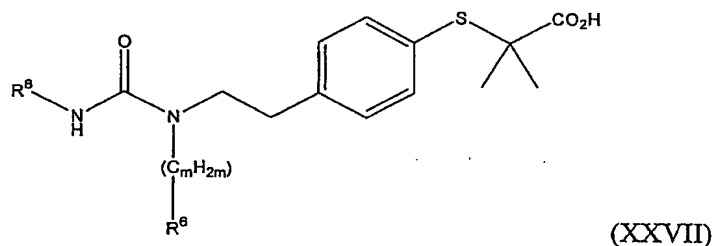
9- or 10-membered heterobicycle which is partially aromatic in more detail is a heterobicycle interrupted by 1, 2, 3, or 4 N heteroatoms;

- 25 substituted 9- or 10-membered heterobicycle which is partially aromatic in more detail is a 9- or 10-membered heterobicycle mono-, di-, tri- or tetrasubstituted independently with hydroxy, oxo, C₁₋₈ alkyl, C₁₋₈ alkoxy, phenyl, phenylC₁₋₈ alkyl or amino substituents;

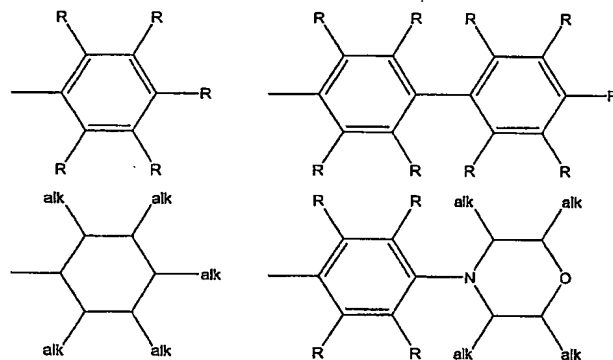
- 30 or a pharmaceutically acceptable acid-addition or base-addition salt thereof.

A dashed line (- -) is none or one double bond between the two carbon atoms.

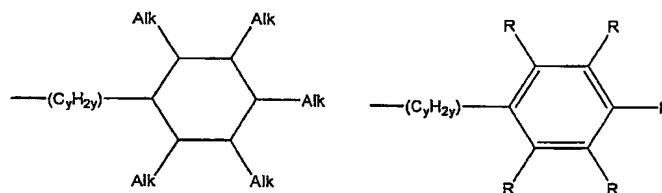
- In yet other embodiments, the therapeutic agent comprises a compound as disclosed
 5 in U.S. Patent No. 6,306,854, e.g., a compound having a structure of Formula
 (XXVII) and esters, salts, and physiologically functional derivatives thereof



- 10 wherein m is from 0 to 20, R⁶ is selected from the group consisting of hydrogen and



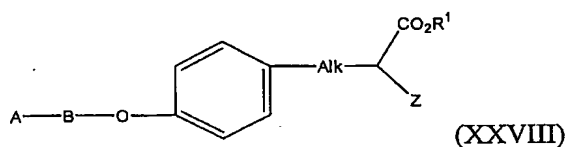
and R⁸ is selected from the group consisting of



- 15 where y is 0, 1, or 2, each alk is independently hydrogen or alkyl group containing 1 to 6 carbon atoms, each R group is independently hydrogen, halogen, cyano, --NO₂, phenyl, straight or branched alkyl or fluoroalkyl containing 1 to 6 carbon atoms and which can contain hetero atoms such as nitrogen, oxygen, or sulfur and which can

contain functional groups such as ketone or ester, cycloalkyl containing 3 to 7 carbon atoms, or two R groups bonded to adjacent carbon atoms can, together with the carbon atoms to which they are bonded, form an aliphatic or aromatic ring or multi ring system, and where each depicted ring has no more than 3 alk groups or R groups that are not hydrogen.

In yet other embodiments of the present invention a therapeutic agent is a compound such as disclosed in U.S. Patent No. 6,294,580 and/or Liu et al., Biorg. Med. Chem. Lett. 11 (2001) 3111-3113, e.g., having a structure within Formula XXVIII:

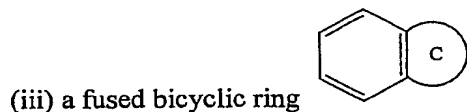


wherein

A is selected from the group consisting of:

(i) phenyl, wherein said phenyl is optionally substituted by one or more of the following groups; halogen atoms, C₁₋₆ alkyl, C₁₋₃ alkoxy, C₁₋₃ fluoroalkoxy, nitrile, or --NR⁷R⁸ where R⁷ and R⁸ are independently hydrogen or C₁₋₃ alkyl;

(ii) a 5- or 6-membered heterocyclic group containing at least one heteroatom selected from oxygen, nitrogen and sulfur; and



wherein ring C represents a heterocyclic group as defined in point (ii) above, which bicyclic ring is attached to group B via a ring atom of ring C;

B is selected from the group consisting of:

(iv) C₁₋₆ alkylene;

5 (v) --MC₁₋₆ alkylene or C₁₋₆ alkyleneMC₁₋₆ alkylene, wherein M is O, S, or --NR² wherein R² represents hydrogen or C₁₋₃ alkyl;

(vi) a 5- or 6-membered heterocyclic group containing at least one nitrogen heteroatom and optionally at least one further heteroatom selected from oxygen,
10 nitrogen and sulfur and optionally substituted by C₁₋₃ alkyl; and

(vii) Het-C₁₋₆ alkylene, wherein Het represents a heterocyclic group as defined in point (vi) above;

15 Alk represents C₁₋₃ alkylene;

R¹ represents hydrogen or C₁₋₃ alkyl;

Z is selected from the group consisting of:

20

(viii) nitrogen-containing heterocyclyl or heteroaryl, e.g., N-pyrrolyl, N-piperidinyl, N-piperazinyl, N-morpholinyl, or N-imidazolyl, optionally substituted with 1-4 C₁₋₆ alkyl or halogen substituents;

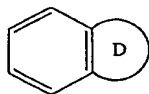
25 (ix) --(C₁₋₃ alkylene) phenyl, which phenyl is optionally substituted by one or more halogen atoms; and

(x) --NR³R⁴, wherein R³ represents hydrogen or C₁₋₃ alkyl, and R⁴ represents C₁₋₆ alkyl, aryl or heteroaryl (e.g., phenyl, pyridinyl, pyrazinyl, pyrimidinyl, pyrrolyl, piperidinyl, piperazinyl, morpholinyl, imidazolyl), optionally substituted by 1-4 C₁₋₆ alkyl, halogen, C₁₋₆alkoxyl, hydroxyl, nitro, cyano, or amino substituents, or --Y--(C=O)--T--R⁵, --Y--SO₂--R⁵, or --Y--(CH(OH))--T--R⁵, wherein:
30

(a) Y represents a bond, C₁₋₆ alkylene, C₂₋₆ alkenylene, C₄₋₆ cycloalkylene or cycloalkenylene, a heterocyclic group as defined in point (vi) above, or phenyl optionally substituted by one or more C₁₋₃ alkyl groups and/or one or more halogen atoms;

(b) T represents a bond, C₁₋₃ alkyleneoxy, --O-- or --N(R⁶)--, wherein R⁶ represents hydrogen or C₁₋₃ alkyl;

(c) R⁵ represents C₁₋₆ alkyl, C₄₋₆ cycloalkyl or cycloalkenyl, phenyl (optionally substituted by one or more of the following groups; halogen atoms, C₁₋₃ alkyl, C₁₋₃ alkoxy groups, C₀₋₃ alkyleneNR⁹R¹⁰ (where each R⁹ and R¹⁰ is independently hydrogen, C₁₋₃ alkyl, --SO₂ C₁₋₃ alkyl, or --CO₂ C₁₋₃ alkyl, --SO₂ NHC₁₋₃ alkyl), C₀₋₃ alkyleneCO₂ H, C₀₋₃ alkyleneCO₂ C₁₋₃ alkyl, or --OCH₂ C(O)NH₂), a 5- or 6-membered heterocyclic group as defined in point (ii) above, a bicyclic fused ring



wherein ring D represents a 5- or 6-membered heterocyclic group containing at least one heteroatom selected from oxygen, nitrogen and sulfur and optionally substituted by (=O), which bicyclic ring is attached to T via a ring atom of ring D: or --C₁₋₆ alkyleneMR¹¹ M is O, S, or --NR¹² wherein R¹² and R¹¹ are independently hydrogen or C₁₋₃ alkyl,

or a tautomeric form thereof, and/or a pharmaceutically acceptable salt or solvate thereof.

Some embodiments of the present invention also include the use of the above compounds for the treatment of Parkinson's disease.

A preferred group of compounds are those of Formula XI, wherein the dotted line represents no bond, R¹ is methyl, X is O and A is O. Especially preferred within this

group are the compounds where R is phenyl, 2-naphthyl and 3,5-bis(trifluoromethyl)phenyl. Another group of preferred compounds are those of Formula XIII, wherein the dotted line represents no bond, R¹ is methyl and A is O. Particularly preferred compounds within this group are compounds where B is CH and R is phenol, p-tolyl, m-tolyl, cyclohexyl, and 2-naphthyl. In alternative embodiments of the present invention, the B is N and R is phenyl.

In still further embodiments, the present invention provides methods for the use of a pharmaceutical composition suitable for administering an effective amount of at least one composition comprising a PPAR γ agonist, such as those disclosed herein, in unit dosage form. In alternative embodiments, the composition further comprises a pharmaceutically acceptable carrier.

Specific examples of compounds of the present invention include, but are not limited to: (+)-5-[[4-[3,4-dihydro-6-hydroxy-2,5,7,8-tetramethyl-2H-1-benzopyran-2-yl)methoxy]phenyl]methyl]-2,4-thiazolidinedione (troglitazone); 4-(2-naphthylmethyl)-1,2,3,5-oxathiadiazole-2-oxide; 5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy]benzyl]-5-methylthiazolidine-2,4-dione; 5-[4-[2-[2,4-dioxo-5-phenylthiazolidin-3-yl]ethoxy]benzyl]thiazolidine-2,4-dione; 5-[4-[2-[N-methyl-N-(phenoxy-carbonyl)amino]ethoxy]benzyl]thiazolidine-2,4-dione; 5-[4-(2-(phenoxyethoxy)benzyl]thiazolidine-2,4-dione; 5-[4-[2-(4-(chlorophenyl)ethylsulfonyl]benzyl]thiazolidine-2,4-dione; 5-[4-[3-(5-methyl-2-phenyloxazol-4-yl)propionyl]benzyl]thiazolidine-2,4-dione; 5-[4-[(1-methylcyclohexyl)methoxy]benzyl]thiadiazolidine-2,4-dione (ciglitazone); 5-[[4-(3-hydroxy-1-methylcyclohexyl)methoxy]benzyl]thiadiazolidine-2,4-dione; 5-[4-[2-(5-methyl-2-phenyloxazol-4-yl)ethoxy]benzyl]thiadiazolidine-2,4-dione; 5-[4-[2-(5-ethylpyridin-2-yl)ethoxy]benzyl]thiadiazolidine-2,4-dione (pioglitazone); 5-[(2-benzyl-2,3-dihydrobenzopyran)-5-ylmethyl]thiadiazoline-2,4-dione (englitazone); 5-[[2-(2-naphthylmethyl)benzoxazol]-5-ylmethyl]thiadiazoline-2,4-dione; 5-[4-[2-(3-phenylureido)ethoxy]benzyl]thiadiazoline-2,4-dione; 5-[4-[2-[N-(benzoxazol-2-yl)-N-methylamino]ethoxy]benzyl]thiadiazoline-2,4-dione; 5-[4-[3-(5-methyl-2-phenyloxazol-4-yl)propionyl]benzyl]thiadiazoline-2,4-dione; 5-[2-(5-methyl-2-

phenyloxazol-4-ylmethyl)benzofuran-5-ylmethyl]-oxazolidine-2,4-dione; 5-[4-[2-[N-methyl-N-(2-pyridyl)amino]ethoxy]benzyl]thiazolidine-2,4-dione (BRL49653); and 5-[4-[2-[N(benzoxazol-2-yl)-N-methylamino]ethoxy]benzyl]-oxazolidine-2,4-dione.

5

In yet other embodiments of the present invention, the therapeutic agents comprise compounds having the structure shown in FIG. 9, wherein: A is selected from hydrogen or a leaving group at the α - or β - position of the ring, or A is absent when there is a double bond between the C $^{\alpha}$ and C $^{\beta}$ of the ring; X is an alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, or substituted alkynyl group having in the range of 2 up to 15 carbon atoms; and Y is an alkyl, substituted alkyl, alkenyl, substituted alkenyl, alkynyl, or substituted alkynyl group having in the range of 2 up to 15 carbon atoms. As used herein, the term "leaving group" refers to functional groups which can readily be removed from the precursor compound, for example, by nucleophilic displacement, under E₂ elimination conditions, and the like. Examples include, but are limited to, hydroxy groups, alkoxy groups, tosylates, brosylates, halogens, and the like.

The therapeutic agents of the present invention (e.g., the compounds in Formulas I-XIII in FIG. 8 and the others described above) are capable of further forming both pharmaceutically acceptable acid addition and/or base salts. All of these forms are within the scope of the present invention.

Pharmaceutically acceptable acid addition salts of the present invention include, but are not limited to, salts derived from nontoxic inorganic acids such as hydrochloric, nitric, phosphoric, sulfuric, hydrobromic, hydriodic, hydrofluoric, phosphorous, and the like, as well as the salts derived from nontoxic organic acids, such as aliphatic mono- and dicarboxylic acids, phenyl-substituted alkanolic acids, hydroxy alkanolic acids, alkanedioic acids, aromatic acids, aliphatic and aromatic sulfonic acids, etc. Such salts thus include sulfate, pyrosulfate, bisulfate, sulfite, bisulfite, nitrate, phosphate, monohydrogenphosphate, dihydrogenphosphate, metaphosphate, pyrophosphate, chloride, bromide, iodide, acetate, trifluoroacetate, propionate,

caprylate, isobutyrate, oxalate, malonate, succinate, suberate, sebacate, fumarate, malcate, mandelate, benzoate, chlorobenzoate, methylbenzoate, dinitrobenzoate, phthalate, benzenesulfonate, toluenesulfonate, phenylacetate, citrate, lactate, maleate, tartrate, methanesulfonate, and the like. Also contemplated are salts of
5 amino acids such as arginate and the like, as well as gluconate, galacturonate, and n-methyl glucamine (See e.g., Berge et al., J. Pharm. Science 66:1 [1977]).

The acid addition salts of the basic compounds are prepared by contacting the free base form with a sufficient amount of the desired acid to produce the salt in the
10 conventional manner. The free base form may be regenerated by contacting the salt form with a base and isolating the free base in the conventional manner or as described above. The free base forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but are otherwise equivalent to their respective free base for purposes of the present
15 invention.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations include, but are not limited to, sodium, potassium, magnesium, calcium,
20 and the like. Examples of suitable amines include, but are not limited to, $N_2 N'$ -dibenzylethylenediamine, chlorprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (See e.g., Berge et al., J. Pharm. Science 66:1 [1977]).

25 The base addition salts of the acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner or as described above. The free acid forms differ from their respective salt forms
30 somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention.

Certain of the compounds of the present invention can exist in unsolvated forms as well as solvated forms, including, but not limited to, hydrated forms. In general, the solvated forms, including hydrated forms, are equivalent to unsolvated forms and are intended to be encompassed within the scope of the present invention. Certain of the compounds of the present invention possess one or more chiral centers and each center may exist in different configurations. The compounds can, therefore, form stereoisomers. Although these are all represented herein by a limited number of molecular formulas, the present invention includes the use of both the individual, isolated isomers and mixtures, including racemates, thereof. Where stereospecific synthesis techniques are employed or optically active compounds are employed as starting materials in the preparation of the compounds, individual isomers may be prepared directly. However, if a mixture of isomers is prepared, the individual isomers may be obtained by conventional resolution techniques, or the mixture may be used as is, with resolution.

Furthermore, the thiazolidene or oxazolidene part of the compounds of Formulas I through XIII can exist in the form of tautomeric isomers, and are intended to be a part of the present invention.

For preparing pharmaceutical compositions from the compounds of the present invention, pharmaceutically acceptable carriers can be in any suitable form (e.g., solids, liquids, gels, etc.). Solid form preparations include, but are not limited to, powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances which may also act as diluents, flavoring agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material. The present invention contemplates a variety of techniques for administration of the therapeutic compositions. Suitable routes include, but are not limited to, oral, rectal, transdermal, vaginal, transmucosal, or intestinal administration; parenteral delivery, including intramuscular, subcutaneous, intramedullary injections, as well as intrathecal, direct intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections, among others. Indeed, it is not

intended that the present invention be limited to any particular administration route.

For injections, the agents of the present invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution,
5 Ringer's solution, or physiological saline buffer. For such transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

In powders, the carrier is a finely divided solid which is in a mixture with the finely
10 dived active component. In tablets, the active component is mixed with the carrier having the necessary binding properties in suitable proportions, which has been shaped into the size and shape desired.

The powders and tablets preferably contain from five or ten to about seventy percent
15 of the active compounds. Suitable carriers include, but are not limited to, magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium carboxymethylcellulose, a low melting wax, cocoa butter and the like, among other embodiments (e.g., solid, gel, and liquid forms). The term "preparation" is intended to also encompass the
20 formation of the active compound with encapsulating material as a carrier providing a capsule in which the active component with or without other carriers, is surrounded by a carrier, which is thus in association with it. Similarly, cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges can be used as solid dosage forms suitable for oral administration.

25

For preparing suppositories, in some embodiments of the present invention, a low melting wax, such as a mixture of fatty acid glycerides or cocoa butter, is first melted and the active compound is dispersed homogeneously therein, as by stirring. The molten homogenous mixture is then poured into convenient sized molds,
30 allowed to cool, and thereby to solidify in a form suitable for administration.

Liquid form preparations include, but are not limited to, solutions, suspensions, and

emulsions (e.g., water or water propylene glycol solutions). For parenteral injection, in some embodiments of the present invention, liquid preparations are formulated in solution in aqueous polyethylene glycol solution. Aqueous solutions suitable for oral use can be prepared by dissolving the active component in water and adding

5 suitable colorants, flavors, and stabilizing and thickening agents, as desired.

Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins, methylcellulose, sodium carboxymethylcellulose, and other well-known suspending agents.

10

Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active component, colorants, flavors, stabilizers, buffers,

15 artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

The pharmaceutical preparation is preferably in unit dosage form. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the

20 active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it can be the appropriate number of any of these in packaged form.

25

The quantity of active component in a unit dose preparation may be varied or adjusted from 0.1 mg to 100 mg, preferably ranging from 0.5 mg to 100 mg according to the particular application and the potency of the active component. The composition can, if desired, also contain other compatible therapeutic agents.

30 General procedures for preparing pharmaceutical compositions are described in Remington's Pharmaceutical Sciences, E. W. Martin ed., Mack Publishing Co., PA (1990).

The assessment of the clinical features and the design of an appropriate therapeutic regimen for the individual patient is ultimately the responsibility of the prescribing physician. It is contemplated that, as part of their patient evaluations, the attending physicians know how to and when to terminate, interrupt, or adjust administration due to toxicity, or to organ dysfunctions. Conversely, the attending physicians also know to adjust treatment to higher levels, in circumstances where the clinical response is inadequate, while precluding toxicity. The magnitude of an administered dose in the management of the disorder of interest will vary with the severity of the condition to be treated, the patient's individual physiology, biochemistry, etc., and to the route of administration. The severity of the condition, may, for example, be evaluated, in part, by standard prognostic evaluation methods. Further, the dose and dose frequency will also vary according to the age, body weight, sex and response of the individual patient.

II. Activity of the Therapeutic Agents

The therapeutic agents described above find use in the treatment of Parkinson's disease. The following description provides examples illustrating the activity of the therapeutic agents.

Experiments conducted during the development of the present invention demonstrate that PPAR γ agonists potently inhibited a diverse range of microglial responses. For instance, one such experiment demonstrates that MPTP-induced dopaminergic cell loss in the substantia nigra pars compacta (SNpc) is attenuated by administration of pioglitazone in a murine model of Parkinson's disease (See e.g. Example 1). Using a similar MPTP-based murine model of Parkinson disease, it was found in other experiments that administration of pioglitazone also attenuated the loss of striatal dopamine (See e.g. Example 2), microglial activation (See e.g. Example 4) and astrocyte activation (See e.g. Example 5).

Other experiments conducted during the development of this invention suggest that indole- and L-tyrosine-based compounds may also be potent PPAR γ agonist and mediators of the neuroinflammatory response. One such experiment examined the

pharmacokinetic profile of 2,3-disubstituted indole 5-phenylacetic acid derivatives in a rat model (See e.g., Example 6). It was noted that one compound of this series, 2-{3-[2-(4-methoxyphenyl)ethyl]-2-phenylindol-5-yl} acetic acid, had an *in vitro* potency at PPAR γ similar to or better than three currently marketed
5 thiazolindinedione antidiabetic agents (i.e., troglitazone, pioglitazone, and rosiglitazone) (See Henke et al., *Biorg. Med. Chem. Lett.* 9:3329-3334 [1999]).

Yet another experiment examined the *in vivo* antihyperglycemic and antihyperlipidemic efficacy of L-tyrosine-based compounds in male Zucker diabetic
10 rats (See e.g., Example 7). One compound in particular, a pyrrole, was found to reduce fasting plasma glucose by 40%, and fasting serum triglycerides by 24% when dosed orally at 10mg/kg bid for 14 days. The experimental data suggest that the novel L-tyrosine-based compound (i.e., the pyrrole) is an even more potent insulin sensitazer than first generation L-tyrosine-based compounds (i.e., farglitazar and
15 GW7845) (See Liu et al., *Biorg. Med. Chem. Lett.* 11:3111-3113 [2001]). Thus, these agents find use as therapeutic agents in the treatment of Parkinson's disease. As discussed above, most of the PPAR γ agonists exhibit substantial bioavailability following oral administration and have little or no toxicity associated with their use (See e.g., Saltiel and Olefsky, *Diabetes* 45:1661 [1996]; Wang et al., *Br. J.*
20 *Pharmacol.* 122:1405 [1997]; and Oakes et al., *Metabolism* 46:935 [1997]).

The present invention provides methods and compositions for attenuating the progressive neurodegenerative processes in Parkinson's disease. However, it is not intended that the present invention be limited to any particular mechanism. Indeed,
25 an understanding of the mechanisms is not necessary in order to practice the present invention.

III. Molecular Regulation of Disease

The present invention provides novel regulatory sequences for controlling COX-2
30 expression, identifying factors that influence COX-2 expression (e.g., drug screening methods), and identifying and controlling signaling pathways responsible for disease states associated with the over- or under-expression of COX-2. In particular, the

present invention provides methods and compositions that utilize the PPAR γ enhancer identified during the development of the present invention. In addition to the inflammatory diseases and conditions described above, the methods and compositions of the present invention find use in a broad array of physiological and cellular events that are influenced by COX-2 expression including, but not limited to, hormone signaling, growth factor signaling, cancer (See e.g., Franzese et al., Melanoma Res. 8:323 [1998]), including colorectal cancer, vision (See e.g., Camras et al., Ophthalmology 103, 1916 [1996]), sleep/wake cycle (See e.g., Scrammell et al., Proc. Natl. Acad. Sci., 95:7754 [1998]), platelet aggregation (See e.g., Wu, J. Formos, Med. Assoc. 95, 661 [1996]), luteolysis (See e.g., Tsai and Wiltbank, Biol. Reprod. 57, 1016 [1997]), cellular differentiation and development, rheumatoid and osteo-arthritis (Vane et al., Ann. Rev. Pharm. Tox. 38:97 [1998]), and hyperalgesia, allodynia, and hyperthermia (Kaufmann, et al., Prostaglandins 54:601 [1997]), among others.

Cyclooxygenase-2, the inducible form of the cyclooxygenase enzyme, is mainly responsible for the pathological effects of prostaglandins where rapid induction of the enzyme would occur in response to such agents as inflammatory agents, hormones, growth factors, and cytokines. Thus, a selective inhibitor of COX-2 will have similar antiinflammatory, antipyretic, and analgesic properties of a conventional non-steroidal antiinflammatory drug, and in addition would inhibit hormone-induced uterine contractions and have potential anti-cancer effects, but will have a diminished ability to induce some of the mechanism-based side effects (e.g., side effects caused by inhibition of COX-1). In particular, in preferred embodiments, such compounds have a reduced potential for gastrointestinal toxicity, a reduced potential for renal side effects, a reduced effect on bleeding times, and in some embodiments, possibly a lessened ability to induce asthma attacks in aspirin-sensitive asthmatic subjects.

The PPAR family has well described roles in adipocytes and serves to regulate the expression of enzymes of lipid metabolism in these cells (See Lemberger et al., Annu. Rev. Cell Dev. Biol. 12:335-363 [1996]; Spiegelman, Cell 93:153-155

[1998]]. An appreciation of the function of these receptors has been substantially enlarged by the recent finding that the PPAR γ isoform is expressed in monocytes and macrophages in which its principal action is to suppress the expression of the proinflammatory cytokines interleukin-1 β (IL-1 β), tumor necrosis factor α (TNF- α), and IL-6 and other proinflammatory products (See Jiang et al., Nature 391:82-86 [1998]; Ricote et al., Nature 391:79-82 [1998]). Importantly, activation of PPAR γ acts to negatively regulate macrophage activation and cytokine expression by antagonizing the activity of the transcription factors NF κ B, AP-1, and STAT proteins (See Lemberger et al., Annu. Rev. Cell Dev. Biol. 12:335-363 [1996]; Ricote et al., Nature 391:79-82 [1998]).

Microglia are the principal immune cell in the brain originating from mesodermally derived macrophages that become permanently resident in the brain during development (See Streit et al., Sci. Am. 273:38-43 [1995]). Like macrophages, microglia respond to various stimuli by acquisition of a reactive phenotype as evidenced by the elevated expression of a number of surface molecules, including major histocompatibility complex class II antigens, CD45, complement receptors MAC-1 and CR4 (See McGeer et al., Glia 7:84-92 [1993]). Activated microglia, like activated macrophages, secrete a diverse range of acute-phase proteins including α -anti-cymotrypsin, α -anti-trypsin, serum amyloid P, C-reactive protein, and complement components, among others (See McGeer et al., Neuro. 42:447-449 [1993]). Importantly, activation of microglia results in the synthesis and secretion of proinflammatory cytokines IL-1 β , IL-6, and TNF- α and the chemokine macrophage chemotactic protein-1 (See McGeer et al., Brain Res. Rev. 21:195-218 [1995]).

The glial reaction is generally considered to be a consequence of neuronal death in neurodegenerative diseases such as Parkinson's disease. In Parkinson's disease, post-mortem examination reveals a loss of dopaminergic neurons in the substantia nigra associated with a massive astrogliosis and the presence of activated microglial cells. Recent evidence suggests that the disease may progress even when the initial cause of neuronal degeneration has disappeared, suggesting that toxic substances released by the glial cells may be involved in the propagation and perpetuation of

neuronal degeneration. Glial cells can release deleterious compounds such as proinflammatory cytokines (TNF- α , IL-1 β , IFN- γ), which may act by stimulating nitric oxide production in glial cells, or which may exert a more direct deleterious effect on dopaminergic neurons by activating receptors that contain intracytoplasmic death domains involved in apoptosis (See Hirsch et al., Ann. N.Y. Acad. Sci. 991:214-228 [2003]).

Experimental

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: N (normal); M (molar); mM (millimolar); μ M (micromolar); mol (moles); mmol (millimoles); μ mol (micromoles); nmol (nanomoles); pmol (picomoles); g (grams); mg (milligrams); μ g (micrograms); ng (nanograms); l or L (liters); ml (milliliters); μ l (microliters); cm (centimeters); mm (millimeters); μ m (micrometers); nm (nanometers); $^{\circ}$ C. (degrees Centigrade); Sigma (Sigma Chemical Co., St. Louis, Mo.), parts per million (ppm).

The following materials and protocols were used in the Examples below.

A. Animals and Treatment

Ten to twelve-week-old male C57BL/6 mice (Janvier, Le Genest St Isle, France), weighing 24–27 g, were housed (two to five animals per cage) with free access to food pellets and water, and were maintained at a constant temperature on a 12-hour light–dark cycle. The animal treatments and care protocols conformed to National Institutes of Health guidelines. Mice received four intraperitoneal injections of MPTP-HCl (15 mg/kg; Sigma Aldrich, St Quentin, France) in saline at 2-hour intervals in 1 day. Control mice received saline only. Animals were killed 2–8 days after the last injection. Pioglitazone (Takeda Chemical Industries, Osaka, Japan) was administered in rodent chow (#5002, Ralston Purina, St Louis, MO, USA) at 120

p.p.m., which was estimated to yield 20 mg/kg/day, beginning 3 days prior to MPTP intoxication, and continuing throughout the entire experiment. This treatment protocol has previously been shown to be useful for investigating PPAR γ mediated effects of pioglitazone (Tang et al. 1999).

5

B. Neurochemical analysis

Determination of dopamine (DA), norepinephrine (NE) and the DA metabolites (DOPAC, HVA) levels in the striatum was performed using high-performance liquid chromatography (HPLC). All structures were homogenized in ice-cold 0.1 M acetic acid containing sodium metabisulphite (10 μ M), EDTA (10 μ M) and ascorbic acid
10 (10 μ M). After centrifugation, the supernatant was passed through a 10 000-MW filter (Nanosep 10 k, Pall). A 50- μ L aliquot of sample was analysed for monoamines and metabolites by isocratic elution and electrochemical detection on a serial electrode array of coulometric flow-through graphite electrodes (Coularray, ESA).
15 Monoamines and metabolites were then identified based on their retention time as well as their electrochemical behaviour across the arrays. The analysis, data reduction and peak identification were fully automated.

C. Western blot analysis

20 For protein extracts, mice were killed by cervical dislocation, and striatum and ventral mesencephalon were rapidly dissected. The samples were homogenized in 10 volumes (w/v) of ice-cold lysis buffer (50 mM TRIS, pH 8; 150 mM NaCl; 1 mM EDTA; 1% NP40) by trituration using a syringe (18- and 26-gauge needles). A cocktail of protease inhibitors, COMPLETE (Roche Molecular Biochemicals,
25 Meylan, France), Pefabloc (1 mM) (Uptima, Montluc, on, France) and pepstatin A (1 μ g/mL) was included in all extractions. Samples of each group were pooled (n = 5) and put on ice for 30 min. After centrifugation (13 000 g, 20 min, 4°C), supernatants were collected and stored at -80°C until analysis. Protein concentrations were determined using the Bradford reagent (Biorad, Hercules, CA, USA). For western
30 blot analysis, samples (200 μ g protein) were loaded on a 10% sodium dodecyl sulfate-polyacrylamide electrophoresis gel. As a positive control, protein extract of rodent adipose tissue (40 μ g per lane) was used. After separation, proteins were

- blotted on a nitrocellulose membrane and subsequently incubated overnight at 4 °C in PBS containing 5% skimmed milk. After washing in phosphate-buffered saline (PBS), membranes were incubated with primary antibody (rabbit polyclonal anti-PPAR γ ; Calbiochem, San Diego, CA, USA; 1 : 1000) in PBS containing 0.05% Tween 20 for 1 h at room temperature. Primary antibody was detected by using an HRP-coupled secondary antibody (anti-rabbit; Jackson Laboratories, San Harbor, MA, USA; 1 : 50 000). Bound secondary antibodies were visualized by using enhanced chemiluminescence (Super Signal west pico; Pierce, Rockford, IL, USA).
- 10 D. Immunohistochemistry
- Animals were anaesthetized with pentobarbital (130 mg/kg; Sigma, St. Quentin, France) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were removed, post-fixed, and cryoprotected. Immunohistochemistry was performed as described previously (Hirsch et al. 1988)
- 15 on free-floating cryomicrotome-cut sections (20 μ m in thickness) encompassing the entire striatum and midbrain. After incubation in 3% H₂O₂/20% methanol, followed by 0.2% Triton X-100 and by 2% bovine serum albumin in 0.1 PBS, the sections were stained overnight at 4°C using a polyclonal antibody against tyrosine hydroxylase (1 : 1000; Pel Freez, Rogers, AR, USA) for dopaminergic neurons, a
- 20 mouse monoclonal antibody against inducible nitric oxide synthase (iNOS; 1 : 250; BD Transduction, Point de Claix, France), a rat antibody against macrophage antigen-1 (Mac-1; 1 : 250; Serotec, Raleigh, NC, USA) for microglia, and a rabbit antibody against glial fibrillary acidic protein (GFAP; 1 : 250; Dako, Trappes, France) for astrocytes. The specificity of these antibodies has already been
- 25 demonstrated and tested by western blot analysis (Viale et al. 1991; Vodovotz et al. 1993; Liberatore et al. 1999). Sections were then treated with secondary antibodies (Vectastain; Vector Laboratory, Burlingame, CA, USA), and subsequently incubated with avidin-biotinylated horseradish peroxidase complex. The peroxidase was revealed by incubation with 0.05% 3,3'-diaminobenzidine tetrahydrochloride
- 30 containing 0.015% hydrogen peroxide, and for iNOS staining 0.16 g/L NiSO₄. For Nissl cell counts TH sections were counterstained with cresyl violet. All sections for a given marker were stained simultaneously for all animals using the same solutions.

E. Cell counting and statistical analysis

Substantia nigra pars compacta (SNpc) TH- and Nissl-positive neuron counts were performed in 20 μm coronal mesencephalic sections 300 μm apart (bregma -2.06 mm to -4.04 mm; Franklin and Paxinos 1996). The total number of process-bearing, TH- and Nissl-stained cells with clearly visible nuclear borders was estimated using a previously described method and an image analysis system (Hunot et al. 1997; Visioscan, BIOCOM, Les Ulis, France). All sections were coded and examined blind. As an index of microglial activation, Mac-1-stained cells with a diameter > 17 μm (cell body) were counted in a similar fashion in the mesencephalon (bregma -2.06 mm to -4.04 mm). For analysis of astrocytes, GFAP-positive cells were counted in a 500 μm \times 500 μm frame overlying the SN at bregma -5.20 mm, and cell number per mm^2 was calculated. In the striatum, measurement of optical density of TH immunostaining as an index of the density of dopaminergic axons and nerve terminals was performed using the same image analysis system. Differences between the four treatment groups (control versus MPTP versus Pioglitazone versus Pioglitazone/MPTP) for TH-, Nissl-, Mac-1-, and GFAP-positive cell counts were analysed using ANOVA, followed by Newman-Keuls's post-hoc analysis or, in the event of failure in normality test, by Kruskal-Wallis' one-way analysis of variance on ranks. In all analyses, the null hypothesis was rejected at the 0.05 level.

EXAMPLE 1

Effect of the PPAR γ Agonist Pioglitazone on TH-Positive Neurons

This example demonstrates that treatment of a mammal with the PPAR γ agonist pioglitazone preferentially prevents the loss of dopaminergic neurons in the SNpc. This experiment was performed on mice treated with MPTP to induce the loss of dopaminergic neurons. The effects of In the SNpc, where the somata of dopaminergic neurons are located, the loss of TH-positive cells in MPTP-treated animals compared with vehicle-treated controls was 23%, 30% and 19% at days 2, 5 and 8 after intoxication, respectively (Fig. 1). The loss of TH-positive neurons was

confirmed by Nissl staining. Pioglitazone prevented the MPTP-induced loss of TH-positive neurons in the SN. In the striatum, the area of projection of dopaminergic neurons from the SN, the MPTP-induced loss of striatal TH immunoreactivity compared with vehicle-treated controls reached 76% at days 2 and 5, and decreased to 53% at day 8 (Fig. 2). In contrast to the protective effect in the SN, pioglitazone had no detectable effect on the loss of TH-positive terminals in the striatum of MPTP-treated animals.

EXAMPLE 2

Effect of the PPAR γ Agonist Pioglitazone on Dopamine and its Metabolites

This example demonstrates that in a mammal treated with MPTP to induce loss of dopaminergic neurons, the loss of dopamine in the striatum is abrogated upon treatment with MPTP. To test striatal nerve terminal injury under pioglitazone treatment in more detail, HPLC measurements for dopamine and its metabolites DOPAC and HVA were performed. Compared with untreated controls, dopamine, DOPAC and HVA baseline contents were reduced by 37%, 48%, and 44% in pioglitazone-treated animals (Figs 3a–c), while there was no significant difference in striatal noradrenaline levels between those two groups (Fig. 3d). In mice that received MPTP, a significant reduction in striatal dopamine, DOPAC, and HVA levels was observed (Figs 3a–c). Striatal dopamine levels were depleted by 85% at day 2, by 84% by day 5, and by 89% by day 8 after MPTP intoxication, compared with 72%, 77%, and 79% in pioglitazone-treated animals, respectively (Fig. 3a). Two days after MPTP intoxication DOPAC levels were reduced by 81%, and HVA levels by 77%, compared with 60% and 43% in the pioglitazone group, respectively (Figs 3b and c).

EXAMPLE 3

Effect of the PPAR γ Agonist Pioglitazone on levels of PPAR γ Protein Levels

This example demonstrates that treatment of a mammal with a Pioglitazone does not alter the levels of PPAR γ protein. Since pioglitazone is a potent agonist of PPAR γ , and this receptor is thought to represent the main pharmacological target of this drug, PPAR γ protein expression was determined in the SN and striatum of animals after MPTP intoxication treated with or without pioglitazone by western blot analysis. PPAR γ was faintly detectable in both SN and striatum compared with the strong expression in the adipose tissue used as control sample, and PPAR γ expression was unaffected by MPTP or pioglitazone treatment at day 2, 5, and 8 after MPTP administration (Fig. 3e).

EXAMPLE 4

Effect of the PPAR γ Agonist Pioglitazone on Microglial Activation

This example demonstrates that application of Pioglitazone to a mammal that has been treated with MPTP reduces the activation of microglial cells in the SNpc. Since PPAR γ agonists are implicated in regulation of microglial cells, we tested the effect of pioglitazone on the glial response. The complement receptor Mac-1 was used as a specific marker of microglia. In saline-injected mice, ramified resting microglia were faintly stained with the Mac-1 antibody in the SN, and to an even lesser extent in the surrounding ventral midbrain (Fig. 4g). Pioglitazone treatment had no effect on resting microglia (Fig. 4h). In MPTP-injected mice, Mac-1 staining in the SN increased markedly after intoxication and this was accompanied by typical morphological changes, such as cell body enlargement, shortening of processes, and loss of ramification (Figs 4a, c and e and Table 1). The MPTP-induced microglial response was maximal at day 2 after MPTP injection and spanned the entire SN, predominating in the SNpc (Fig. 4a). At day 5 post MPTP, the total number of cells with activated morphology was reduced. The remaining cells showed an increased staining intensity and an irregular shape, characteristic of phagocytic cells, and their anatomical distribution was more confined to the region of the SNpc (Fig. 4c). At

day 8, Mac-1 expression was no longer different from control sections (Fig. 4e, Table 1). In pioglitazone-treated mice, there was a strong reduction of Mac-1 expression at day 2 post MPTP, and no difference in Mac-1 staining in comparison to control sections thereafter (Figs 4b, d and f and Table 1). MPTP also induced the expression of iNOS in the SN, revealing cells with cytoplasmic staining and morphology of activated microglia at day 2 post MPTP injections (Fig. 3i), which were absent in pioglitazone-treated animals (Fig. 4j). Fewer iNOS-positive cells than Mac-1-positive cells were found in the SN of MPTP-treated animals. Since the anatomical distribution and morphology of iNOS immunoreactive cells was virtually identical to that of Mac-1-positive microglia, no double staining was performed. In the striatum, MPTP intoxication led to a transient increase of Mac-1 expression with a similar time course as that of microglial activation observed in the ventral mesencephalon (Fig. 5). In contrast to the midbrain, pioglitazone had no effect on striatal Mac-1 expression (Fig 5).

Table 1:

	Day 2	Day 5	Day 8
MPTP	1596 ± 289	711 ± 355	0 ± 0
MPTP + pioglitazone	185 ± 78***	0 ± 0	0 ± 0

MPTP-induced microglial activation in the ventral. Total number of Mac-1-stained cell bodies with a diameter >17 μ m per ventral mesencephalon was estimated as an index of microglial activation 2, 5 and 8 days after MPTP intoxication. Activated cells were confined to the region of the SN, as determined by TH immunoreactivity of adjacent mesencephalic sections. The cell counts between the two groups (MPTP vs. MPTP + pioglitazone) were compared using a nonparametric statistical test because distribution differed significantly from normality. Data are represented as means ± SEM (n = 5 per group). No Mac-1-stained cells with a diameter >17 μ m were detected in control animals, with or without pioglitazone treatment.

***Significant difference (p < 0.001) between pioglitazone treated group and control group.

EXAMPLE 5**Effect of the PPAR γ Agonist Pioglitazone on Astrocytes**

5

This example demonstrates that application of Pioglitazone to a mammal that has been treated with MPTP reduces the levels of activated astrocytes in the SNpc. MPTP increased the number and size of cells expressing GFAP in the entire SN, but predominately in the SNpc (Fig. 6, Table 2). The density and staining intensity of GFAP expressing cells in the SN was maximal at day 5 post MPTP treatment compared with vehicle-treated controls and decreased thereafter. GFAP-immunoreactive cells in MPTPtreated animals shared the characteristics of reactive astrocytes, i.e. labelled astrocytes were hypertrophic with shortening and thickening of cytoplasmic processes (Fig. 6c). Pioglitazone treatment attenuated the MPTP-induced increase in GFAP-positive astrocytes in the mesencephalon by 59% at day 2, 36% at day 5, and 53% at day 8 (Table 2), and GFAP-positive cells were rather evenly distributed in the SN of pioglitazone-treated animals compared with the MPTP-treated group (Fig. 6).

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As in the mesencephalon, MPTP intoxication led to an astrocytic response in the striatum, estimated by the increased number of GFAP expressing cells with a reactive morphology, which was maximal 5 days after the treatment (Fig. 7). In the striatum, pioglitazone showed no effect on MPTP-induced changes in GFAP expression (Fig. 7).

25 Table 2:

	Day 2	Day 5	Day 8
Saline	41 \pm 3		
Saline + pioglitazone	41 \pm 8		
MPTP	115 \pm 31**	173 \pm 16***	94 \pm 15*
MPTP + pioglitazone	47 \pm 7†	110 \pm 9‡	44 \pm 8

Table 2 Density of glial fibrillary acidic protein (GFAP)-positive cells in the substantia nigra. The number of GFAP stained perikarya per mm² in the region of the SN (at bregma -5.20 mm) was estimated as an index of astrogliosis 2, 5 and 8 days after MPTP intoxication. Data are represented as means \pm SEM (n \approx 5-7 per group). *, **, *** Significant difference (p < 0.05, 0.01, 0.001, respectively) between MPTP-treated and saline- injected control group. †, ‡ Significant difference (p < 0.05/0.01, respectively) between the time matched MPTP group and MPTP + pioglitazone-treated group.

EXAMPLE 6

5 Synthesis and Biological Activity of a Novel Series of Indole-derived PPAR γ Agonists

This example demonstrates that a novel series of indole 5-carboxylic acids (e.g., Formula XVII wherein R⁷ is carboxy or carboxyalkyl) bind and activate PPAR γ .

- 10 PPARs play an important role as regulators of lipid and glucose metabolism. One particular subtype, PPAR γ , is predominately expressed in adipose tissue and plays a pivotal role in adipocyte differentiation. In an effort to discover novel activators of the PPAR family, this experiment described the synthesis and structure-activity of a series of 2,3-disubstituted indole 5-acetic acids. The general preparation of most
- 15 analogs described in this experiment utilized a Fischer indole synthesis of the appropriately substituted ketone and aryl hydrazine, followed by simple functional group transformation if needed. All synthesized compounds were tested for their affinity and potency of PPAR activation. Even at the highest concentration tested (10 μ M), all compounds were devoid of affinity and functional activity at the
- 20 PPAR α and PPAR δ subtypes. One compound in particular, 2-{3-[2-(4-methoxyphenyl)ethyl]-2-phenylindol-5-yl}acetic acid, displayed strong *in vitro* potency and selectivity at PPAR γ . This compound displayed excellent pharmacokinetics in the rat, with a low mean total body clearance (CL_{TOT} = 3 mL/min/kg) and good half-life ($t_{1/2}$ = 3.8 h). The compound's mean oral

bioavailability in the rat was 78% dosed as a solution and 73% dosed as a suspension. The compound's pharmacokinetic profile suggests that it might be a suitable candidate for oral administration.

5 EXAMPLE 7

Synthesis and Biological Activity of L-Tyrosine-based PPAR γ Agonists with Reduced Molecular Weight

- 10 This example demonstrates *in vivo* activity of an L-tyrosine-based pyrrole analog in a rodent model of Type 2 diabetes. See Liu et al., *Biorg. Med. Chem. Lett.* 11 (2001) 3111-3113. Potent PPAR γ agonists such as GW7845 and farglitazar have been reported (See B. Henke et al., *J. Med. Chem.* 41:5020-5036 [1998]). These compounds, however, contain asymmetric centers and are not as potent as the S-
- 15 enantiomers, which are synthesized from naturally occurring L-tyrosine. Through a process of model building into the PPAR γ crystal structure, alternative N-substituents were selected that would add a small lipophilic substituent while mimicking some of the effects of the intramolecular hydrogen bond present in the benzophenone analogue. Compounds were built with the PPAR γ binding site by
- 20 removal of the benzophenone group from farglitazar, and growth of alternative substituents onto the tyrosine nitrogen, using the MVP program. The growth process was carried out twice, once within the protein binding site and once in aqueous solution. Finally, a pyrrole synthesized from L-tyrosine was 10 times more potent at PPAR γ and 5 times more potent at PPAR α than its enantiomer (synthesized
- 25 from D-tyrosine). The *in vivo* antihyperglycemic and antihyperlipidemic efficacy of the pyrrole (synthesized from L-tyrosine) were evaluated in male Zucker diabetic fa/fa rats ($n=6$). When dosed orally at 10 mg/kg bid for 14 days, the pyrrole reduced fasting plasma glucose by 40% and fasting serum triglycerides by 24%, while HDL-cholesterol was raised by 31% compared to vehicle treated animals.
- 30 These data demonstrate that L-tyrosine based derivatives may be potent PPAR γ agonists.

Claims:

1. A method for treating Parkinson's disease, comprising administering a therapeutically effective amount of at least one PPAR γ agonist to a subject.
5
2. The method of claim 1, wherein said PPAR γ agonist is selected from thiazolidinedione and thiazolidinedione derivatives.
3. The method of claim 2, wherein said thiazolidinedione derivatives are
10 selected from troglitazone, ciglitazone, pioglitazone, rosiglitazone, englitazone, GW7845 and farglitazone.
4. The method of claim 1, wherein said PPAR γ agonist is selected from indole-derived compounds, indole 5-carboxylic acid derivatives, and 2,3-disubstituted
15 indole 5-phenylacetic acid derivatives.
5. The method of claim 1, wherein said PPAR γ agonist is an L-tyrosine-derived compound.
- 20 6. The method of claim 1, wherein said subject is selected from subjects identified as being susceptible to Parkinson's disease and subjects suffering from Parkinson's disease.
7. The method of claim 2, wherein said therapeutically effective amount of said
25 thiazolidinedione and thiazolidinedione derivatives is between 0.1 mg to 100 mg.
8. The method of claim 1, wherein said agonist is administered orally.
9. A method of reducing the activation of microglial cells in the substantia
30 nigra pars compacta of a subject, comprising administering to said subject a therapeutically effective amount of at least one PPAR γ agonist.

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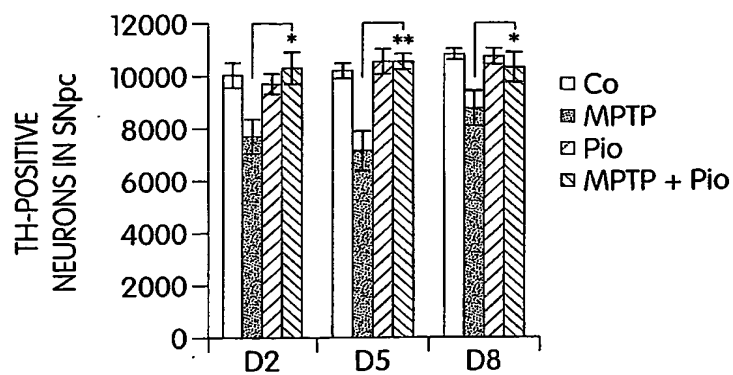


Fig. 1A



Fig. 1B



Fig. 1C

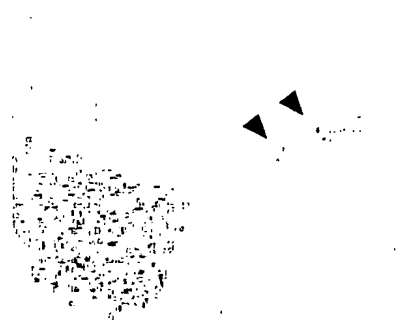


Fig. 1D



Fig. 1E

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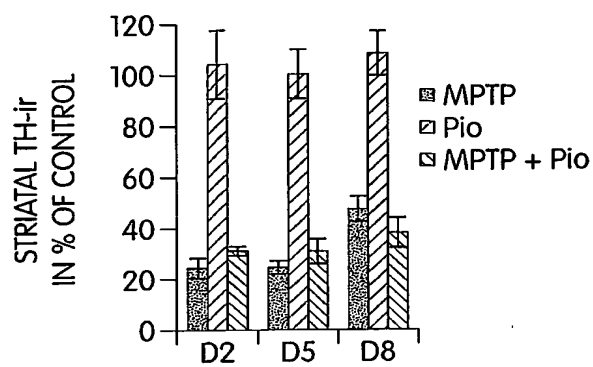


Fig. 2A



Fig. 2B

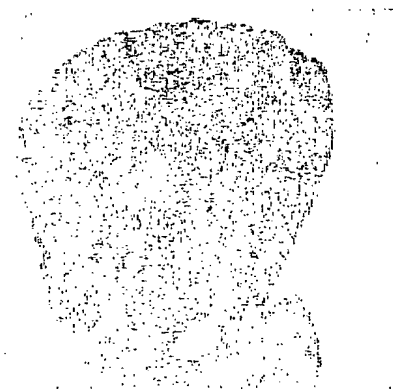


Fig. 2C

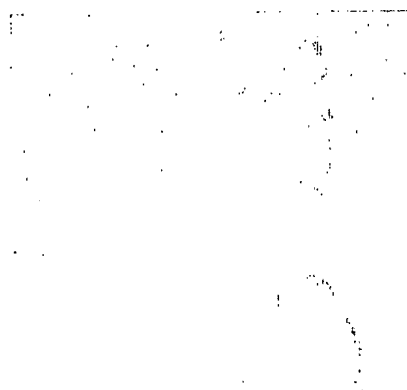


Fig. 2D

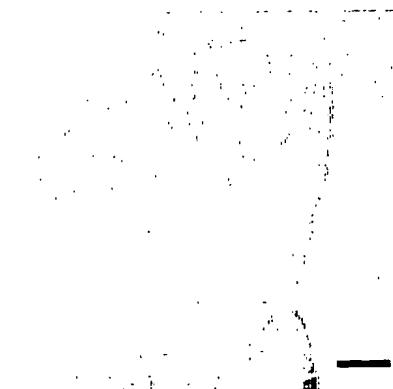


Fig. 2E

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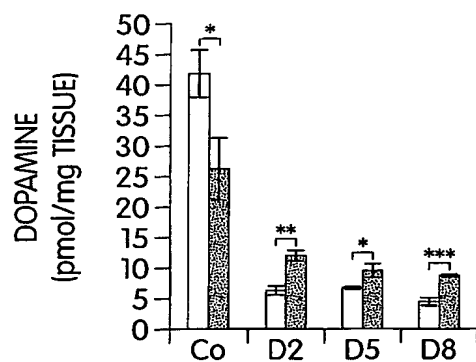


Fig. 3A

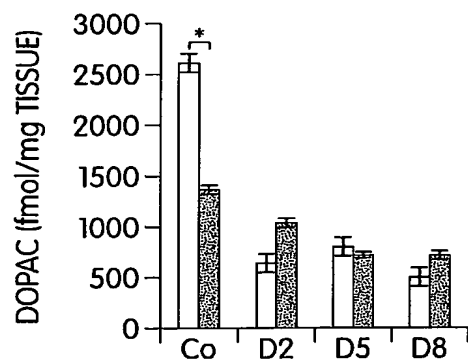


Fig. 3B

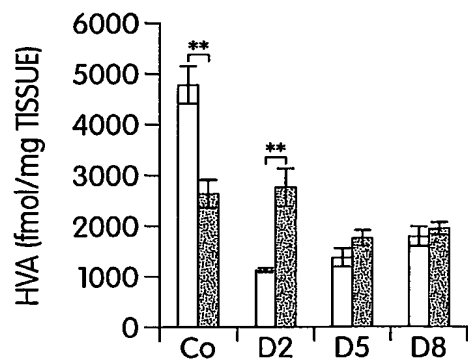


Fig. 3C

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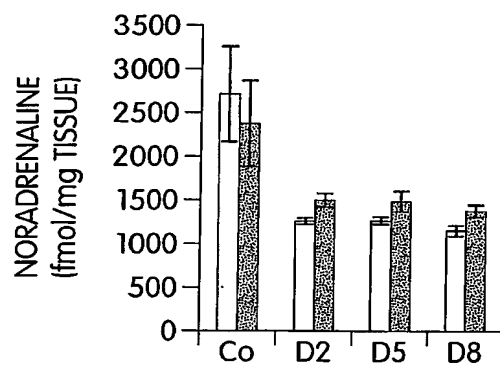


Fig. 3D

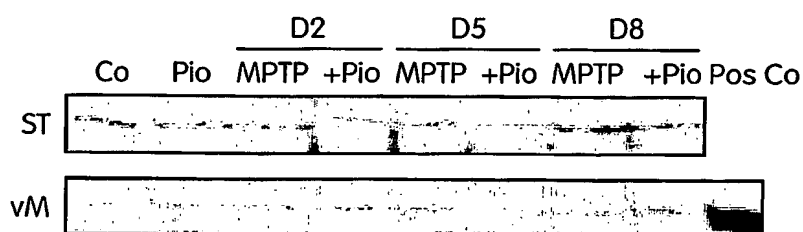


Fig. 3E



Fig. 4A



Fig. 4B

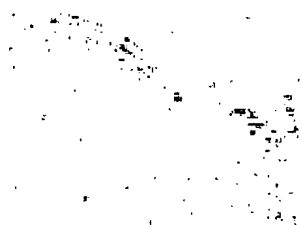


Fig. 4C



Fig. 4D

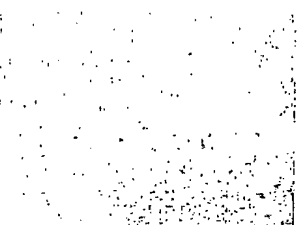


Fig. 4E



Fig. 4F

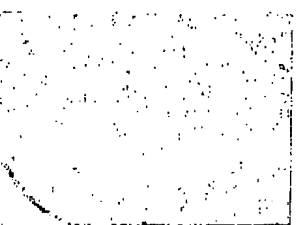


Fig. 4G



Fig. 4H

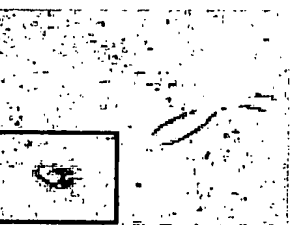


Fig. 4I

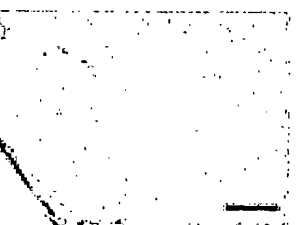


Fig. 4J

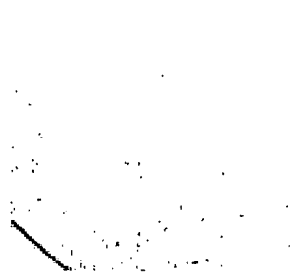


Fig. 5A

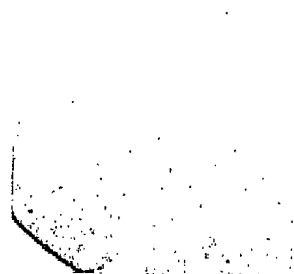


Fig. 5B



Fig. 5C



Fig. 5D

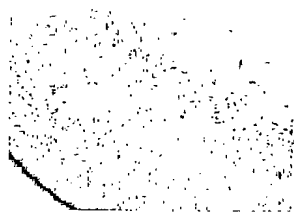


Fig. 5E



Fig. 5F

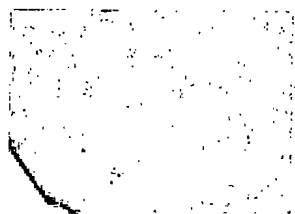


Fig. 5G



Fig. 5H

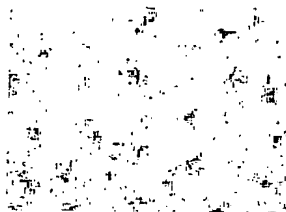


Fig. 6A



Fig. 6B

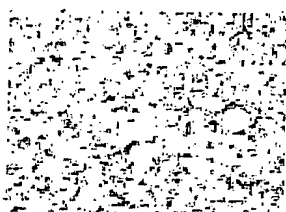


Fig. 6C



Fig. 6D

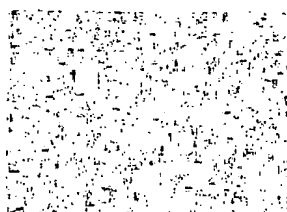


Fig. 6E

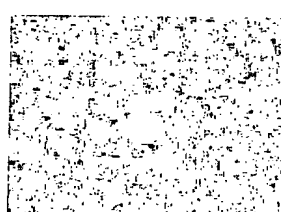


Fig. 6F



Fig. 6G

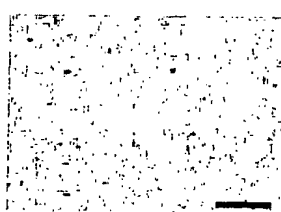


Fig. 6H

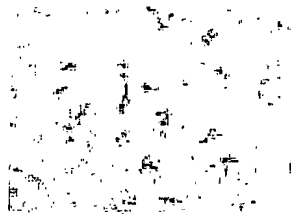


Fig. 7A



Fig. 7B

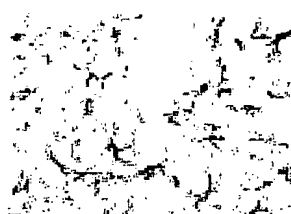


Fig. 7C



Fig. 7D

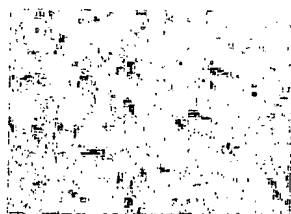


Fig. 7E

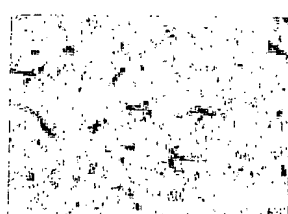


Fig. 7F



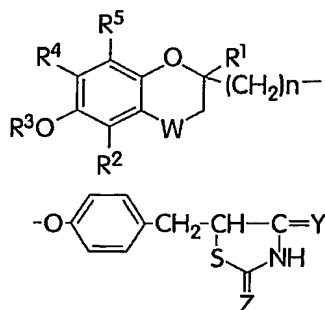
Fig. 7G



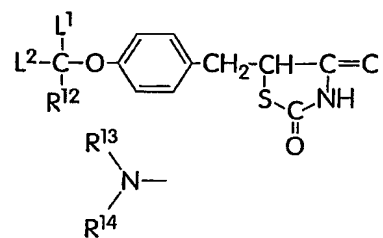
Fig. 7H

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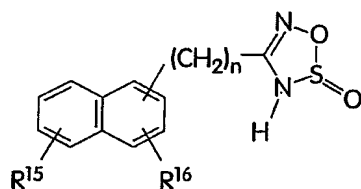
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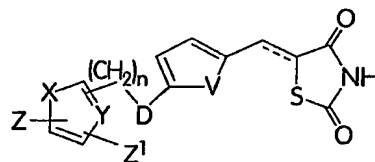
FORMULA II

POSSIBLE FORMULA USED FOR R¹¹

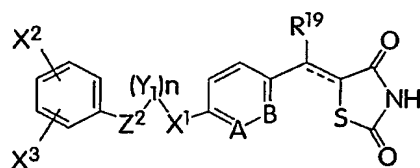
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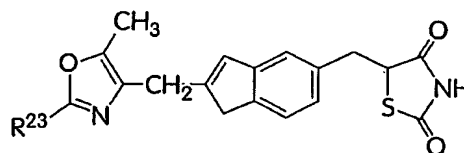
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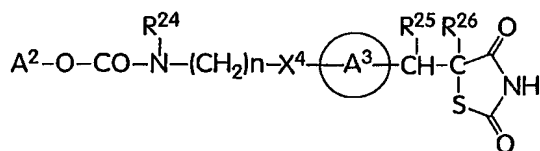
FORMULA V



FORMULA VI



FORMULA VII



FORMULA VIII

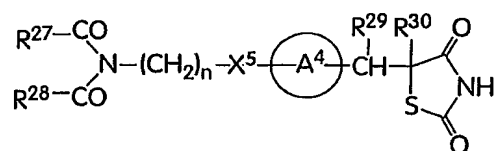
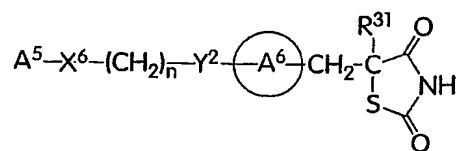


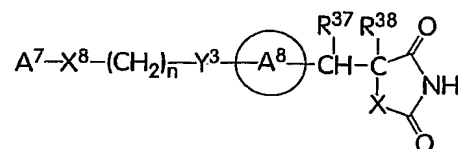
Fig. 8

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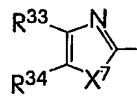
FORMULA IX



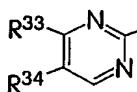
FORMULA X



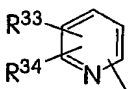
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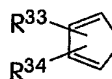
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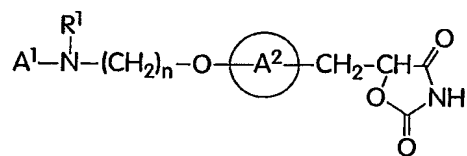
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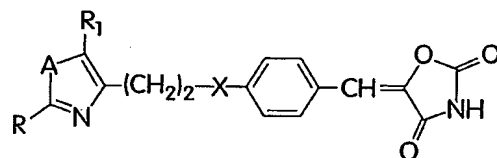
FORMULA (d)



FORMULA XI



FORMULA XII



FORMULA XIII

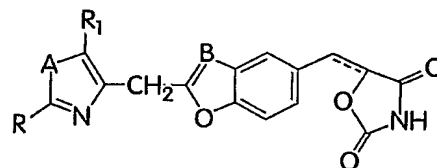


Fig. 8 Cont.

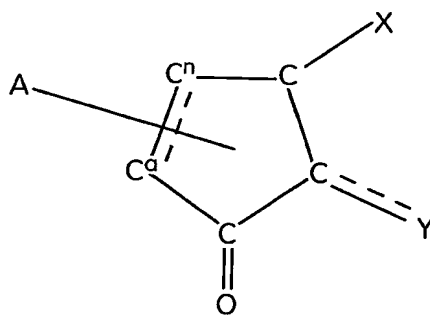


Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US03/23082

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A61K 31/425, 31/50, 31/497, 31/44, 31/42
US CL : 514/369, 252.05, 254.02, 256, 342, 375, 376

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/369, 252.05, 254.02, 256, 342, 375, 376

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HIRSCH et al. The Role of Glial Reaction and Inflammation in Parkinson's Disease, June 2003, Volume 991, pages 214-228, especially page 224..	1-9
X	WO 02/49626 A2 (SMITHKLINE BEECHAM P.L.C.) 27 June 2002 (27.06.2002), see entire document, especially page 2, lines 1-40, page 8, lines 33-38.	1-8
—		9
A		
X	BRIEDERT et al. Protective Action of the Peroxisome Proliferator-Activated Receptor-Gamma Agonist Pioglitazone in a Mouse Model of Parkinson's Disease, 2002, Journal of Neurochemistry, Volume 82, Pages 615-624. See entire document, especially page 615, column 1 to page 616, column 1 and page 621, column 1 to column 2. +.	1-9
X	US 6,369,098 B1 (PERSHADSINGH et al) 09 April 2002 (09.04.2002) see abstract, see column 12, lines 24-37, column 20, line 23 to column 24, line 54, column 27, lines 28-57.	1-8
—		9
Y		

☐ Further documents are listed in the continuation of Box C.

☐ See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"E" earlier application or patent published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

14 December 2003 (14.12.2003)

Date of mailing of the international search report

09 JAN 2004

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Facsimile No. (703)305-3230

Authorized officer

Donna Jagoe

Telephone No. (703) 308-1235

INTERNATIONAL SEARCH REPORT

PCT/US03/23082

Continuation of Item 4 of the first sheet:

The title does not meet PCT Rule 4.3 because it is not properly describe the invention. The new title is "Methods for the Treatment of Parkinson's Disease".

Continuation of B. FIELDS SEARCHED Item 3:

WEST 2.1, STN, file cluster MEDICINE, Search terms, PPAR gamma agonist, thiazolidinedione, parkinson's, microglial, tyrosine.